# THE MECHANISM OF UPTAKE AND INTRACELLULAR FATE OF LEUPEPTIN IN RAT YOLK SACS

BY

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## Abbreviations

| | |
|---|---|
| c.p.m. | counts per minute |
| d.p.m. | disintegrations per minute |
| $^{125}$I-BSA$_{fd}$ | formaldehyde-denatured $^{125}$I-iodine-labelled albumin |
| $^{125}$I-PVP | $^{125}$I-iodine-labelled polyvinylpyrrolidone |
| TCA | trichloroacetic acid |
| E-64 | (N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine) |

Standard chemical symbols and abbreviations for amino acids have been used where appropriate throughout the thesis.

## Abstract

The aim of the work described in this thesis was to determine the mode of uptake of the tripeptide proteinase inhibitor leupeptin, in order to better interpret the effect of leupeptin on protein degradation within cells.

Different methods of leupeptin detection were developed to monitor various aspects of the accumulation and action of leupeptin within yolk-sac tissue. The rate of accumulation, intracellular fate, and degree of inhibition of intracellular proteinases afforded by leupeptin, were all determined.

The major route of entry of leupeptin into yolk sacs appeared to be fluid phase pinocytosis, though other modes, such as permeation, could not be entirely dismissed. Intracellular active leupeptin reached a steady state concentration that was capable of fully inhibiting lysosomal cathepsins B+L, though did not completely inhibit degradation of $^{125}I\text{-}BSA_{fd}$. Leupeptin was able to permeate into isolated intact lysosomes.

# CHAPTER 1

# GENERAL INTRODUCTION

# GENERAL INTRODUCTION

## 1.1. Protein Degradation

In mammals proteolysis occurs both intracellularly and extracellularly, and ranges from the cleavage of a single peptide bond to the total hydrolysis of a protein.

The function of proteolysis varies greatly. Total hydrolysis of proteins is important in the degradation of dietary protein to provide nutrients for an organism, in turnover (ie synthesis and degradation) of essential endogenous proteins within an organism to maintain the integrity of such proteins (by removing damaged proteins or wrongly synthesised proteins), and in controlling the quantities of regulatory enzymes and of cell receptors through degradation. Limited proteolysis, involving cleavage of one or two peptide bonds is important in, for example, the activation of enzymes in enzyme cascade systems (eg in the clotting, and the complement systems of blood), and in the production of the active forms of peptide hormones and enzymes by proteolytic removal of an oligopeptide fragment from pro-hormones and zymogens. The functions of various types of hydrolysis are discussed by Barrett (1978), Dean (1980) and Holzer & Heinrich (1980).

Proteinase enzymes have been purified and characterized from a wide variety of intracellular and extracellular sources (eg see Barrett & McDonald, 1980). They are generally classified according to their site of cleavage and mode of catalytic action:-

a) Exoproteinases (or exopeptidases, depending on whether the substrate is a protein or peptide) cleave peptide bonds one or two residues away from the ends of a polypeptide chain.

b) Endoproteinases (and endopeptidases) can cleave bonds distant from the ends of a polypeptide chain. Endoproteinases may be further classified, according to their mechanism of catalysis and active-site residues, into thiol, aspartate, serine and metalloendoproteinases. (This method of classification is discussed by Barrett, 1977.)

The various mechanisms and pathways of extracellular proteolysis have, in general, been well characterised. The proteinases involved are generally readily isolated, thus their specificity and kinetics can be determined. For example, the major enzymes responsible for proteolysis in the gut, ie trypsin, chymotrypsin and pepsin, can be readily purified and their properties have been studied in detail. The roles of endogenous inhibitors of proteolysis in controlling such processes as clotting and fibrinolysis are beginning to be understood.

Comparatively little is known about the mechanism(s) and pathway(s) of intracellular protein degradation. Many intracellular proteinases have been isolated (though purification is more difficult than for extracellular enzymes) and characterised (see Barratt & McDonald, 1980). They are often classified according to their location within the cell rather than by their catalytic action. Thus intracellular proteinases may be classified as 'lysosomal' or 'non-lysosomal'. Lysosomes are membrane-bound vesicles containing, together with other hydrolytic enzymes, a wide variety and high concentration of proteinases collectively capable of hydrolysing virtually any protein presented to them. Lysosomal proteinases have pH optima that are slightly acidic (pH 4-5). 'Non-lysosomal' proteinases have been isolated from many sites in the cell, eg plasma membrane, mitochondria, ribosomes, peroxisomes, endoplasmic reticulum (Holzer & Heinrich, 1980). Many of these proteinases are highly specific for a particular cleavage site or protein. A pH optimum

near 7.0 is common to many non-lysosomal proteinases.

Proteins that are degraded intracellularly may be classified according to their origin and rate of turnover:-

a) Exogenous proteins are extracellular proteins that are taken into the cell by endocytosis before being degraded; such proteins include serum proteins, proteins derived from micro-organisms, and hormones. Exogenous protein uptake and degradation are important functions of certain cells such as mononuclear phagocytes, cells of the reticuloendothelial system, hepatocytes and renal cells.

b) Endogenous proteins are those of which the cell itself is composed. They are generally divided into three classes according to their rate of turnover (see Hershko & Ciechanover, 1982).

i) Long-lived proteins, which have a 'long' half-life (the actual half-life varies considerably), and are thought to include structural proteins and proteins of cell organelles.

ii) Short-lived proteins, which have shorter half-lives and include regulatory proteins (enzymes, receptors, etc.) and some soluble proteins.

iii) Abnormal proteins, which are produced by errors during synthesis, and possibly by damage to the protein during its lifetime in the cell. (It is not known whether the latter abnormal proteins are treated in the same way as those proteins with synthetic errors.) These proteins are thought to be degraded very rapidly.

A number of workers have postulated that intracellular protein degradation occurs via at least two pathways, either distinct from or interconnected to each other, and there is now much evidence to support this hypothesis, eg see reviews by Ballard (1978), Dean (1980) and Hershko & Ciechanover (1982). One pathway of degradation is known as the lysosomal pathway, in which proteins enter the

lysosomes and are degraded by the lysosomal proteinases. It is thought that all exogenous proteins, most long-lived endogenous proteins, some secretory proteins, and all proteins degraded during periods of enhanced proteolysis are degraded by the lysosomal pathway. The other pathway, generally known as the non-lysosomal pathway, is less well characterised. Proteinases specific for certain functions have been found throughout cells, but it is not clear how they might contribute to the overall degradation of cell proteins. It is thought that non-lysosomal proteolysis is important in the degradation of abnormal and short-lived proteins. Possibly only the initial, rate-limiting step in the degradation is non-lysosomal, with the final stages of proteolysis being completed within lysosomes.

Most of the investigations into mechanism(s) of intracellular proteolysis and types of protein degraded by them have been made using inhibitors of proteolysis. The use of these inhibitors is discussed in the next section.

## 1.2 Inhibitors of Protein Degradation

Inhibitors of protein degradation and of proteinases are used to determine which proteolytic pathway or individual proteinases are responsible for the degradation of specific proteins and classes of proteins eg Neff *et al.* (1979), Cockle & Dean, (1982) Grinde & Seglen (1980). The inhibitors used are chosen either to be specific for one particular type of proteinase (eg thiol endoproteinases) or to act generally on the entire lysosomal pathway. (No inhibitors that affect only non-lysosomal proteolysis have yet been identified.) Inhibitors are added to cell or tissue cultures, or administered to whole animals, and the rate of degradation of specific proteins or classes of protein monitored. Any decrease would suggest that

degradation normally occurs via the inhibited pathway or involves a particular proteinase.

The most commonly used inhibitors in the study of intracellular proteolysis can be divided into two main groups namely the lysosomotropic amines and the 'peptide inhibitors'. An excellent review of both types is given by Seglen (1983).

Lysosomotropic amines are weak bases that are uncharged at neutral pH, but become protonated at acid pH. They accumulate within lysosomes since they permeate across the lysosomal (and plasma) membranes in their neutral form, become protonated in the acidic lysosomal lumen and are then unable to permeate back across the membrane in their charged forms. They act by buffering the lysosomal pH away from the optimum required by lysosomal proteinases, thus are thought to affect all lysosomal proteinases (de Duve et al., 1974; Poole & Ohkuma, 1981).

Peptide inhibitors include a wide range of natural and synthetic peptides that are thought to act as transition-state analogues. They generally contain modified or entirely novel amino acids. For example, the carboxylic acid group of the carboxy-terminal amino acid may be replaced by an aldehyde (eg leupeptin, antipain), a diazomethane (eg Z-Phe-Ala-$CHN_2$), or a chloromethyl ketone group (eg Phe-Arg-$CH_2Cl$). The inhibitors pepstatin and E-64 contain the unusual residues 4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA) and epoxysuccinyl-leucylamido-(4-guanidino)butyrate, respectively. Peptide inhibitors are usually specific for a particular class of proteinase.

In order for the results of inhibition studies to be interpreted unequivocally, a number of important factors must be considered:-

i) The specificity of the inhibitor must be known. If an inhibitor is used to block a particular enzyme or pathway, it should not

affect other proteinases or proteolytic pathway(s) within the cell. Ideally, it should also not affect any other process within the cell. Unfortunately, these requirements for specificity are not met by many of the commonly-used inhibitors. For example, lysosomotropic amines are known to cause non-specific toxic effects, affecting energy metabolism, protein synthesis, membrane fusion etc. (Dean *et al.*, 1984; Seglen, 1983). They are, however, thought to be specific for the lysosomal pathway of protein degradation, due to their mechanism of inhibition (ie increase of pH within lysosomes). Conversely, the majority of peptide inhibitors are non-toxic *in vivo* and *in vitro* (Umezawa & Aoyagi, 1977; Grinde & Seglen, 1980) and do not affect processes other than proteolysis. (An exception is the chloromethyl ketones, which are cytotoxic). However, peptide inhibitors are not necessarily specific to a particular proteinase or pathway. Depending on the access of the inhibitor to different intracellular sites, several or all proteinases of a particular class may be inhibited. It is therefore very important to know the sites to which peptide inhibitors have access within the cell, and the location of their possible target enzymes. Problems associated with this are discussed by Dean (1979); Cockle & Dean (1982); Hershko & Ciechanover (1982), and McGowan *et al.* (1976).

ii) The degree of inhibition afforded by the inhibitor should be assessed. If inhibition of a particular proteinase/pathway is incomplete, an estimate of the amount of protein degradation occurring via other route(s) will be too high because the inhibited pathway will continue to contribute to proteolysis. Lysosomotropic agents are generally assumed to inhibit lysosomal degradation totally, since all amines give about the same degree

of inhibition of endogenous protein degradation and their effects are not additive when more than one amine is used (Grinde & Seglen, 1980; Seglen, 1983). This assumption can be challenged. For example, a constant percentage of proteolytic activity may remain within the lysosomes even at the increased pH; addition of more than one amine may not give rise to any further increase in lysosomal pH. The potency of the peptide inhibitors depends on a number of factors, such as the affinity ($K_i$) of the inhibitor for its target enzyme(s), and the intracellular concentration of the inhibitor (which itself depends on the rate of uptake and metabolic fate of the inhibitor). Complete inhibition of the lysosomal pathway is not expected to be achieved by any one peptide inhibitor, due to their specificity for particular classes of proteinases. Generally, a cocktail of inhibitors with different specificities is used to give more complete inhibition of the lysosomal cathepsins eg Grinde & Seglen (1980), McGowan et al. (1976). However, since peptide inhibitors may be capable of inhibiting non-lysosomal proteinases, such cocktails cannot be assumed to inhibit only lysosomal proteolysis.

Further knowledge of the sites of action and potency as inhibitors of proteolysis within intact cells would greatly enhance the interpretation of results obtained using peptide inhibitors, and may aid their use as therapeutic agents for the treatment of tissue-wasting diseases (in which the rate of intracellular proteolysis is increased and the rate of protein synthesis decreased; Griggs & Rennie, 1982). One peptide inhibitor that is commonly used in investigating tissue proteolysis, and which has been tested for therapeutic use in the tissue-wasting disease muscular dystrophy, is leupeptin. The main characteristics of this inhibitor are described

in the following section.

## 1.3 Leupeptin

Leupeptin is a tripeptide that is synthesised by various species of Streptomycetes. It is a mixture of propionyl- and acetyl-L-leucyl-L-leucyl-L-arginal; occasionally L-valine or L-isoleucine may be substituted for L-leucine (Kondo et al., 1969; Kawamura et al., 1969). Arginal is an arginine residue, but with an aldehyde group in place of the usual terminal carboxylic acid group. Leupeptin exists predominantly in a cyclic or hydrated form (Maeda et al., 1971) but the active structure is probably the aldehyde (see Fig. 1.1). The aldehyde group acts as a transition-state analogue and the peptide moiety provides enzyme specificity. Leupeptin inhibits the major lysosomal thiol endoproteinases, cathepsins B and L, and several other lysosomal cathepsins eg H, N, S and T (Seglen, 1983). It also inhibits non-lysosomal proteinases such as the thiol endoproteinases calpain, also known as calcium-activated neutral proteinase (Suzuki et al., 1981) and tissue kallikrein (Barrett & McDonald, 1980). Various extracellular enzymes are also inhibited by leupeptin, including papain (which is a thiol endoproteinase) trypsin, chymotrypsin, plasma kallikrein, and plasmin (which are all serine endoproteinases); see Umezawa & Aoyagi (1977). The precise mechanism of interaction of leupeptin with trypsin has been described (Kuramachi et al., 1979). Degradation of all exogenous proteins examined to date is quite strongly inhibited by leupeptin; eg see Dunn et al. (1979), Grinde & Seglen (1980), Neff et al. (1979); Knowles et al. (1981). Degradation of endogenous proteins is also inhibited, but the degree of inhibition observed varies considerably between different tissues and/or incubation regimes. Experiments in which the degradation of both long-lived and short-lived or abnormal

proteins were investigated separately showed that leupeptin inhibited degradation of long-lived proteins but not short-lived or abnormal proteins (Neff et al., 1979).

In experiments designed to test its use as a therapeutic agent to treat muscular dystrophy, leupeptin has been added to various types of dystrophic and normal muscle tissue cultured in vitro, or administered in vivo to genetically dystrophic chickens (which are used as a laboratory model of muscular dystrophy) and the degree of atrophy of the tissue monitored by electron microscopy. Most workers found the leupeptin provided protection against atrophy of dystrophic tissue in vitro, and found that the ultrastructure and integrity of normal tissue were maintained for longer than for controls incubated without leupeptin (Libby & Golberg, 1978; Libby et al., 1979; McGowan et al., 1976). In vivo results have been varied. Some workers claim leupeptin is able to delay or prevent the onset of muscular dystrophy if the inhibitor is administered before the onset of symptoms of the disease (Sher et al., 1981; Stracher et al., 1978). Other workers have found leupeptin to have little or no effect in vivo (Enomoto & Bradley, 1977).

Leupeptin has a number of limitations in its use. It does not totally inhibit the lysosomal pathway of degradation, since it does not inhibit cathepsin D, or exoproteinases. (It is occasionally used in conjunction with other inhibitors eg the cathepsin D inhibitor pepstatin, to give a greater degree of inhibition). It may also inhibit non-lysosomal enzymes, therefore it cannot automatically be used to distinguish lysosomal from non-lysosomal pathways of degradation. Its potency against each leupeptin-sensitive enzyme is not fully established in vivo. Only cathepsin B inhibition has been specifically measured (Neff et al., 1979); the degree of inhibition of other lysosomal or non-lysosomal enzymes has not been determined.

Notably, the degree of inhibition of cathepsin L, which is the most powerful lysosomal proteinase (Barrett & Kirschke, 1981) has not been measured *in vivo*.

It is not fully established how leupeptin gains access to intracellular sites, or to which sites it has access (as noted by Libby & Goldberg, 1978; McGowan *et al.*, 1976). (Some more recent studies suggest leupeptin is able to diffuse across membranes, but evidence for this is only circumstantial; see Section 1.4). There is some evidence that it can be degraded and/or inactivated by enzymes present in certain tissues (Beynon *et al.*, 1981; Brown & Beynon, 1983) and it is not known whether the steady-state intracellular concentration of leupeptin is sufficient to give maximal inhibition of leupeptin-sensitive enzymes.

This thesis attempts to answer certain questions surrounding the intracellular site of action, potency, and intracellular fate of leupeptin. The mode of uptake of leupeptin was studied in order to predict likely site(s) of action, and the intracellular fate and potency were determined by monitoring the quantity of active leupeptin, and activity of cathepsin B + L within the tissue, respectively.

## 1.4 Possible Mechanisms of Uptake of Leupeptin into Tissues

It is important to investigate the mechanism of uptake of leupeptin not only because knowledge of this characteristic will provide information about the intracellular location(s) to which leupeptin has access but also because very little is known about the mechanism of uptake of small peptides by cells. There are two main routes by which small extracellular molecules may enter cells: namely pinocytosis and permeation across the plasma membrane.

Pinocytosis is the process of membrane invagination and

'pinching-off' to form vesicles containing extracellular material (eg low mol. wt. solutes, soluble macromolecules, colloids). Substrates taken up by pinocytosis may be ingested entirely within the droplet of extracellular fluid (fluid-phase pinocytosis) or may become adsorbed to the plasma membrane (adsorptive pinocytosis). Characteristics of fluid-phase and adsorptive pinocytosis are given in reviews by Silverstein et al. (1977) and Pratten et al. (1980). Uptake of macromolecular substrates by pinocytosis leads to the material being enclosed in membrane-bound vesicles, which then fuse with lysosomes. The substrate remains trapped within the lysosomal system unless it can be degraded into smaller units (eg amino acids, monosaccharides, etc.) that are able to permeate through the lysosomal membrane (Lloyd, 1969; 1971).

Uptake by membrane permeation occurs by one or more of three possible mechanisms. Passive diffusion across the phospholipid bilayer of the plasma membrane, or possibly through small pores, may occur for small and/or lipophilic molecules. Alternatively, entry could occur by facilitated diffusion and/or active transport which involve specific carriers in the membrane. Active transport requires energy, and, unlike passive or facilitated diffusion, can transport substrates across the membrane against a concentration gradient. Characteristics of membrane permeation mechanisms are described by West (1983). Uptake of substrates by permeation across the plasma membrane would result in delivery of the substrate to the cytosol. Permeation across the lysosomal membrane may then follow, provided the lysosomal membrane has similar permeability properties (transport systems, etc.) to the plasma membrane.

Thus, if leupeptin cannot cross the plasma or lysosomal membranes and is taken up by pinocytosis, it will become concentrated within

lysosomes and inhibit lysosomal enzymes alone in a specific manner. However, if leupeptin is taken up both by pinocytosis and by membrane permeation, it would have access to non-lysosomal and to lysosomal enzymes. In both cases, the location of leupeptin can only be predicted provided the permeability of lysosomal and plasma membranes are similar, as is suggested by Hales et al. (1984).

Methods are known that permit a distinction to be made between fluid and adsorptive pinocytosis (Pratten et al., 1980), and between passive, facilitated, or active membrane permeation (West, 1983). Unfortunately, it is difficult to distinguish between pinocytosis and membrane permeation since similar properties are observed for both modes of uptake. (For example, both pinocytosis and active transport have an energy requirement and saturation kinetics could apply to adsorptive pinocytosis and to facilitated or active transport.) Workers investigating pinocytosis generally use large macromolecules (eg proteins or synthetic polymers), colloids (eg colloidal gold) or molecules to which the plasma membrane is considered to be impermeable (eg sucrose, inulin). Workers in the field of membrane permeation generally study small molecules (eg amino acids, dipeptides, monosaccharides) or ions (eg $Na^+$, $K^+$, $Ca^{2+}$) and incubate tissue or cells for short periods (eg 2 min) to preclude any major contribution towards uptake by pinocytosis.

Both pinocytosis and direct membrane permeation have been proposed by different workers to explain the uptake characteristics of tripeptides.

Pinocytosis was suggested by Ehrenreich & Cohn (1969) to be the mechanism of entry of $(D\text{-}Ala)_3$ into macrophages. Their conclusion that uptake was by pinocytosis was based on measurements of the lysosomal location of the tripeptide, and on the effects of inhibitors of pinocytosis (2,4 DNP and parafluorophenylalanine) on its

uptake. Shaw & Dean (1980) suggested that uptake of the peptide proteinase inhibitor Z-Phe-Arg-$CHN_2$ was by adsorptive pinocytosis, on the basis of measurements both of the rate of uptake of a radiolabelled derivative, and of displacement of the labelled inhibitor from the cell membrane by unlabelled Z-Phe-Ala.

Although tripeptides are generally considered too large and hydrophilic to cross membranes by passive diffusion, several transport systems are known for small peptides in various mammalian tissues and in bacteria (Matthews, 1977). It is now well-established that gut mucosa contains fairly non-specific, active transport system(s) for dipeptides (Burston et al., 1977) that are distinct from amino acid carriers (Silk, 1977). Tripeptides (but not tetrapeptides or above) appear to be taken up by the dipeptide transport system; substitution of the terminal amine or carboxyl groups of the peptide abolishes affinity for transport. Transport of peptides has also been reported in kidney (Ganapathy, 1981), brain (eg Inoue et al., 1984), liver, skeletal muscle, erythrocytes, and tumour cells (Matthews & Payne, 1975). The specificity of transport in these tissues and cells is not well documented; often only specific, biologically-active peptides have been studied.

Uptake of peptide proteinase inhibitors has been investigated by a few workers, although conclusions tend to be based on the action of the inhibitor, which may not give a good indication of the rate or mode of uptake. Dean (1979) reported that pepstatin (isovaleric acid-L-Val-L-Val-AHMHA-L-Ala-AHMHA) entered macrophages via pinocytosis since it acts very slowly on intracellular proteolysis (similar results were observed in fibroblasts and hepatocytes). Z-Phe-Ala-$CHN_2$ also acts quite slowly and is thought to enter macrophages via adsorptive pinocytosis (Shaw & Dean, 1980). The inhibitor E-64 was found to become incorporated into the cytosolic

fraction of liver before the lysosomal fraction (Hashida *et al.*, 1982), which was interpreted as uptake by permeation. Several reports suggest that leupeptin (Ac-Leu-Leu-Argal) may also enter by permeation (Cockle & Dean, 1984; Borin *et al.*, 1981) since it is able to inhibit intracellular degradation very rapidly (Seglen *et al.*, 1979; Dean, 1979). However, no in-depth study of the mode of entry of this inhibitor has been made to date.

Uptake of leupeptin into rat visceral yolk sac (a mammalian tissue whose pinocytic behaviour is well characterised *in vitro*) is reported in the work described in this thesis.

## 1.5 Rat Visceral Yolk-Sac Tissue

The rat visceral yolk sac was used as a model tissue to study the uptake and intracellular fate of leupeptin. The rat yolk-sac incubation technique has several advantages over other commonly used methods of studying uptake (such as various routes of administration to living animals, organ perfusion, cell culture). It is easy to set up *in vitro* short-term incubation of the tissue and results are generally reproducible. Loss by filtration of low mol. mass molecules by the kidneys, that occurs *in vivo*, is avoided. Unlike *in vivo* studies only one type of endocytic cell, the yolk-sac epithelial cell, is involved. Also, previous investigations indicate that tissue integrity and cell structure are largely maintained. This contrasts with many cells isolated by methods involving exposure to proteolytic enzymes.

Another advantage in the use of yolk-sac tissue is that it is very active proteolytically. It has been suggested that one physiological role of the yolk-sac is to supply nutrients to the fetus via uptake and degradation of macromolecules (Freeman *et al.*, 1981). The degradation of endogenous and exogenous proteins have

been studied previously in the yolk sac, using both peptide inhibitors and lysosomotropic amines as inhibitors (Livesey et al., 1980; Knowles et al., 1981). Leupeptin was found to be quite effective, inhibiting the degradation of an exogenous protein (formaldehyde-denatured $^{125}$I-labelled albumin) by 80% and endogenous protein (mostly long-lived) by about 20% (Knowles et al., 1981).

The visceral yolk sac is one of the placental membranes that, in vivo, surround the developing fetus. It is comprised of three cell layers separated by two basal membranes. The outer cell layer, which after 16 days gestation is exposed to the uterine cavity, is known as the visceral endoderm. It is comprised of columnar vitelline epithelial cells, that are 'cemented together' by tight-junctions. They possess a highly microvillous apical surface, with calveoli extending into the cytoplasm, and are very pinocytically active. The cell membrane is coated with a mucopolysaccharide glycocalyx. The cells of the visceral endoderm are attached to the visceral basement membrane. The middle cell layer is known as the mesenchymal cell layer, through which run vitelline blood capillaries. Below the mesenchymal cells is the serosal basement membrane, to the lower side of which is attached the third, thin, layer of mesothelial cells. About one-third of the visceral yolk sac (nearest the chorioallantoic placenta) is highly folded (macrovillous); the cell layers are the same in both villous and non-villous regions (see Fig. 1.2).

## 1.6 Aims of the work described in this thesis.

It was hoped that the mode(s) of entry of leupeptin into cells could be determined in order to interpret more fully the action of this inhibitor on intracellular proteolysis. The mode of uptake of leupeptin was investigated to give an indication of the initial intracellular location, and the permeability of the lysosomal

membrane towards leupeptin monitored to determine whether leupeptin had free access to both lysosomal and non-lysosomal sites once within the cell. The optimum incubation period with leupeptin to achieve maximal inhibition of leupeptin-sensitive lysosomal enzymes was established. The intracellular fate of leupeptin was also assessed by measuring the amount of active inhibitor within the tissue.

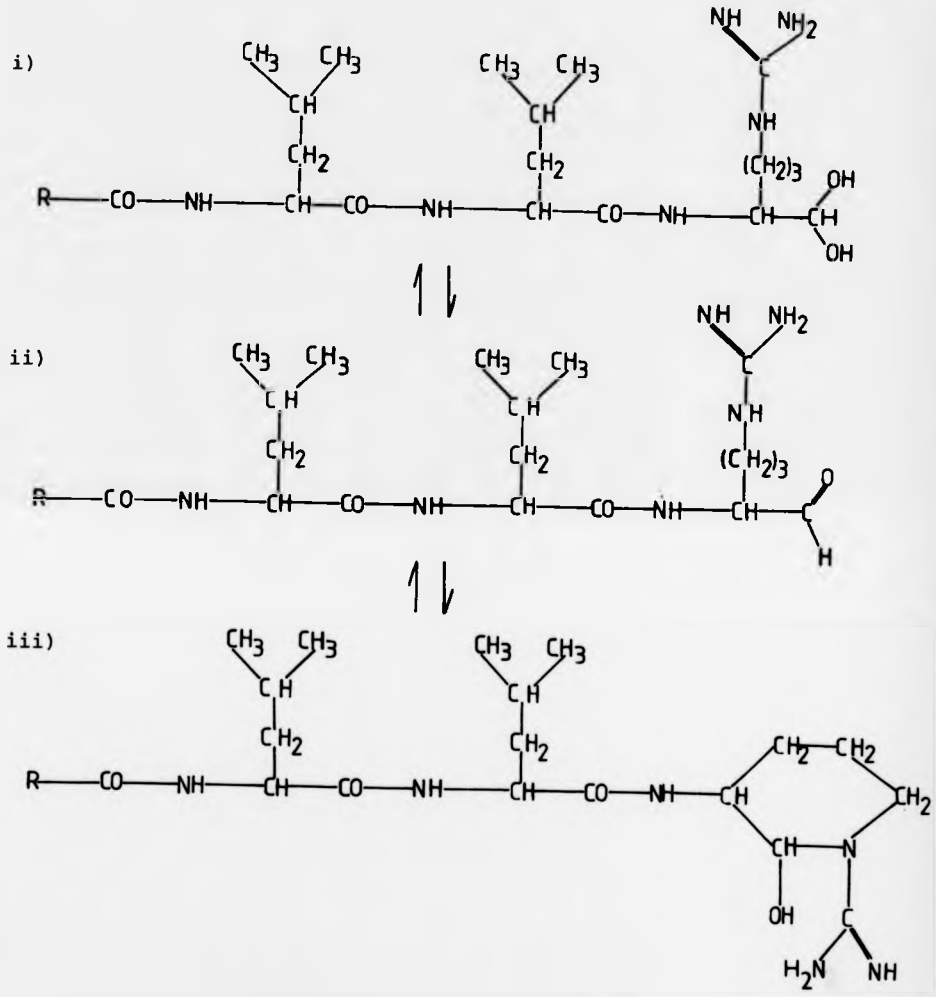


Fig. 1.1 Different Hydration States of Leupeptin

i) Hydrated form

ii) Aldehyde form

iii) Cyclic form

From Maeda et. al. (1971)

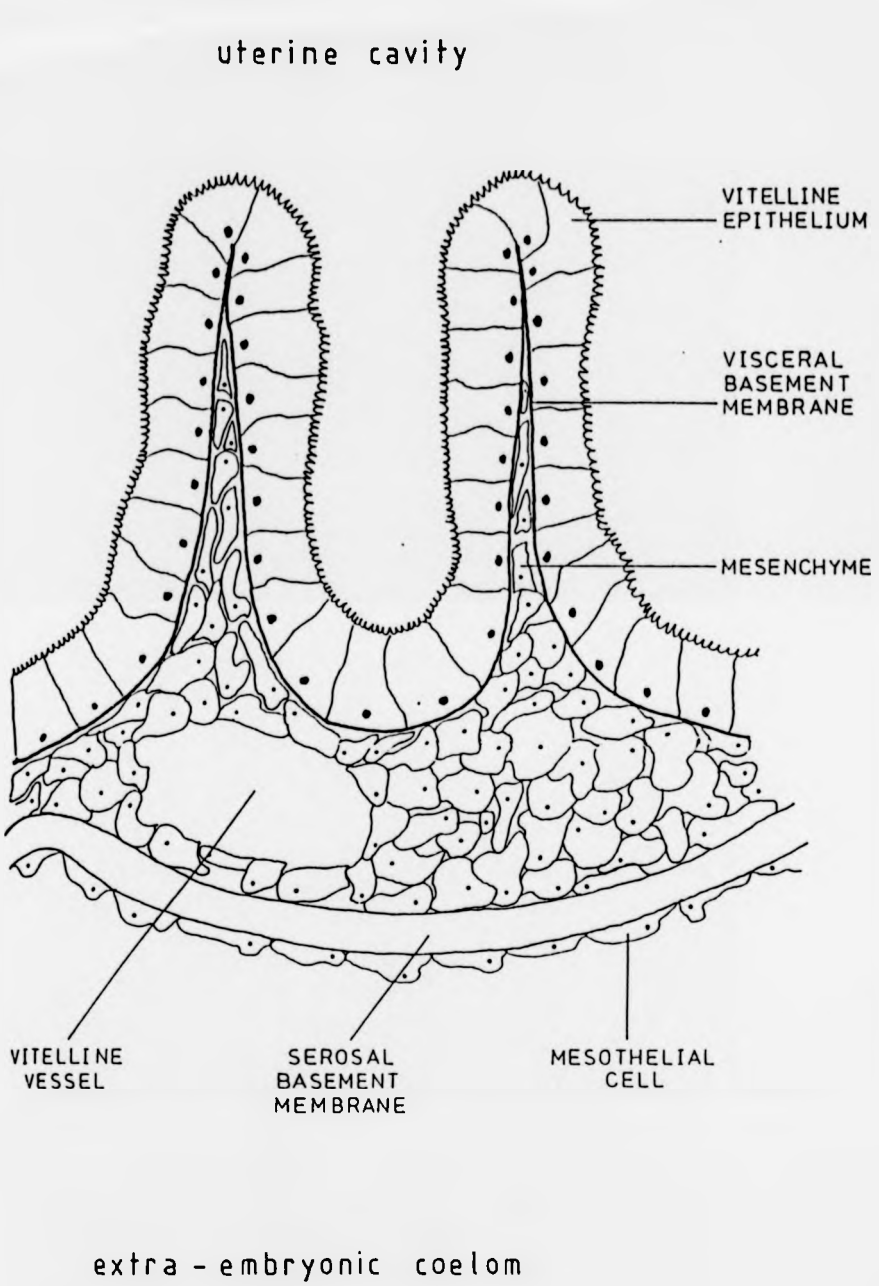


Fig. 1.2 Transverse section through a highly folded region of the rat yolk sac

(From Ibbotson, G. Thesis, 1977)

# CHAPTER 2

# GENERAL MATERIALS AND METHODS

## GENERAL MATERIALS AND METHODS

### I. MATERIALS

#### Equipment

| | |
|---|---|
| Gamma Counters | 5142 Selektronic Gamma Spectrometer.<br>Packard Instrument Ltd., Caversham, Berks., U.K. |
| | 5136 Auto Gamma Scintillation Spectrometer.<br>Packard Instument Ltd., Caversham, Berks., U.K. |
| Beta Counter | 2425 Tri Carb Liquid Scintillation Spectrometer.<br>Packard Instruments Ltd., Caversham, Berks, U.K. |
| Spectrophotometer | CE373 Linear Readout Grating Spectrophotometer.<br>Cecil Instruments Ltd., Cambridge, U.K. |
| Water Baths | Thermal Laboratory Equipment.<br>A Searle Co., Oldham, U.K. |
| Centrifuge | Mistral 4L.<br>M.S.E., London, U.K. |
| Homogenizers | Hand-held ground-glass; Jencons Scientific Ltd.<br>Hemel Hempstead, Herts., U.K. |
| | Potter Elvehjem-type; Tri-R Instruments. |

| | |
|---|---|
| | Rockville Instruments, N.Y., U.S.A. |
| LP3 tubes and scintillation vial inserts | L.I.P. Shipley, W. Yorkshire, U.K. |
| Calculator | TI 55. Texas Instruments. |
| Computer | GEC 4080 Main-frame computer. |

**Chemicals**

| | |
|---|---|
| Medium 199 | Powdered medium 199, with Earl's Salts and L-glutamine; containing 100,000 units Penicillin-Streptomycin. Gibco Europe Ltd., Uxbridge, Middlesex, U.K. |
| 95% $O_2$:5% $CO_2$ Gas | British Oxygen Co. Ltd., Medical Gases Division. London, U.K. |
| Calf Serum | Calf Serum 1, Heat Inactivated. Wellcome Reagents Ltd., Beckenham, U.K. |
| $^{125}I^-$ | Code IMS.30. Sodium iodide in dilute sodium hydroxide, pH 7-11 (free from reducing agents), approximately 1mCi per 10µl. |

Amersham International PLC, Bucks, U.K.

| | |
|---|---|
| $^{125}$I-PVP | Code IM.33P. $^{125}$I-Polyvinylpyrrolidone in succinate buffer solution. Concentration 2-4mg/ml. [The solution as supplied was generally diluted to 20-40µg/ml in medium 199 before addition to flasks to give a final concentration of 2-4µg/ml.] Amersham International PLC, Bucks, U.K. |
| Leupeptin | Ac-L-Leu-L-Leu-Arginal.$0.5H_2SO_4.H_2O$ Peptide Institute Inc., Minoh-shi, Osaka 562, Japan. |
| Trypsin | Type IX (code T-0134), from porcine pancreas. Sigma Chemical Company, Poole, Dorset, U.K. |
| Bovine Serum Albumin | Code A-7906 Sigma Chemical Co., Poole, Dorset, U.K. |

All other chemicals were of analytical grade and were generally obtained from Sigma Chemical Company, Poole, Dorset, U.K. or from BDH Chemicals Ltd., Poole, Dorset, U.K.

## II. METHODS

### 2.1. Preparation of Formaldehyde-Denatured $^{125}$I-Labelled Bovine Serum Albimin ($^{125}$I-BSA$_{FA}$)

2.1a. **Iodination Procedure** Bovine serum albumin (BSA) was iodinated using the chloramine-T method adapted from the method described by Williams *et al.* (1971) which, in turn, was based on the method of Bocci (1969). Albumin (20mg) was dissolved in 0.05M $Na_2H$-$KH_2$ phosphate buffer, pH 8.0, (9ml), and cooled on ice. A solution of $^{125}$I-iodide (1mCi in 10µl) was then added. After thorough mixing, a solution of choramine-T (4ml, 1mg/ml solution in buffer) was added and the reaction mixture stirred for exactly 8 min. The reaction was then stopped by the addition of sodium metabisulphite solution (3ml, 2mg/ml in buffer). Excess iodide (in the form of solid KI, approximately 500mg) was then added to aid displacement of unreacted $^{125}$I-iodide during subsequent dialysis.

2.1b **Denaturation Procedure.** A formaldehyde solution (10%, w/v) was prepared by mixing 5ml formalin (ie 40% formaldehyde) with 15ml of 0.5M sodium carbonate buffer (pH10). The iodination reaction mixture (undialysed) was mixed with an equal volume of 10% formaldehyde, and stirred at room temperture for approximately 70h. The solution was then transferred to Visking tubing (leaving approximately 50 µl for analysis of labelling efficiency) and dialysed against at least three changes of 1% sodium chloride solution (5l), until the dialysis solution showed background radioactivity. The contents of the Visking tubing were then transferred into bottles in 5 ml aliquots and stored at -20°C.

2.1c **Labelling Efficiency.** The labelling efficiency was determined using the reserved undialysed reaction mixture.

The solution (approximately 50µl) was made up to approximately 5ml with distilled water and samples (1ml) assayed for radioactivity. Serum (0.1ml) and TCA (0.5ml, 20%w/v solution) were added to precipitate the protein-bound $^{125}I$, this precipitate was spun down at 1000g for 20 min. The TCA-soluble fraction (mostly $^{125}I$-iodide) was transferred to another tube for counting. The labelling efficiency was calculated:-

$$\text{Labelling Efficiency} = \frac{(T-S)}{T} \times 100$$

Where T = Total radioactivity in 1ml sample, (c.p.m., corrected for background).

and S = TCA-soluble radioactivity (c.p.m. in the same sample, corrected for background and counting geometry - see p25).

After dialysis the amount of TCA-soluble material in the preparation was determined by treating a small (50µl) sample of dialysed solution in the same way as described above. The percentage of TCA solubles was calculated:-

$$\%\ \text{sols} = \frac{\text{TCA-soluble c.p.m. (corrected)}}{\text{Total c.p.m.}} \times 100$$

This value was typically 0.4-0.6% at the time of bottling.

## 2.2. Method for Incubation of Rat Yolk-Sac Tissue

The method was based on that of Williams *et al.* (1975a,b); modifications of this method will be described in Sections 2.2b. and 2.6.

Pairs of Wistar rats from an inbred colony were mated overnight in grid cages. If a sperm-plug was detected beneath the cage the next morning, pregnancy was timed from midnight on the night of mating. At 17.5 days of gestation, the pregnant females were sacrificed by asphixiation in carbon dioxide, then the uterus was removed and placed in warm (37°C) medium 199 in a petri-dish. A longitudinal cut was made in the uterus wall, and each conceptus removed. For each conceptus, the placental cap and foetus were dissected from the yolk sac. The yolk sacs were then placed in fresh medium in a clean petri-dish and each gently separated from the under-lying amnion. The yolk sacs were then placed in sterile, 50ml Erlenmeyer flasks (cleaned by sonication in $Na_2CO_3$ solution) containing medium 199 (with or without 10% calf serum) under a 95% oxygen 5% carbon dioxide atmosphere, each sealed with a sterile silicone rubber bung. The flasks and yolk sacs were maintained at 37°C in a shaking water-bath for at least 10 minutes before addition of substrate. An aliquot (10% v/v of total volume of medium in flask) of substrate dissolved in medium was added to each flask, the flasks regassed with 95% $O_2$: 5% $CO_2$, and the yolk sacs incubated for up to 9h in the water bath. After various incubation periods, yolk sacs were removed from the flasks and washed in three changes of ice cold 1% saline (approximately 20ml, 3 x 2min). A sample of incubation medium was removed from the flask at the same time as the yolk sac. The precise method of analysing these samples depended on the experiment and type of substrate used, see Sections 2.4 and 2.5, below.

2.2a. **Standard Volume Incubation Method** The following details of the general incubation technique apply to the standard volume method. As described in Williams *et al.* (1975a,b), yolk sacs were incubated singly in separate flasks, in a total volume of 10ml medium.

The yolk sacs were added to flasks containing 9ml substrate-free, gassed medium, then 1ml of a solution of substrate in medium 199 added to initiate the time-course of incubation. At each sample time (1-9h) two flasks were removed from the water-bath, the yolk sacs rinsed and duplicate 1 ml samples of medium taken from each flask.

2.2b **Reduced Volume Incubation Method** This method was developed to conserve scarce/expensive materials. It is suitable for substrates that are not captured rapidly from the medium and are thus not markedly depleted from the medium during the course of an experiment. Yolk sacs were incubated together (3 per flask) in a final volume of 7ml medium.

The three yolk sacs were added to a flask containing 6.3ml substrate-free, gassed medium then substrate dissolved in medium (0.7ml) was added. At each sample time the shaking mechanism on the water bath was stopped, and one yolk sac removed from each of two different flasks, together with 1ml medium from each flask; the flasks remaining in the water-bath throughout. The flasks were then regassed, re-stoppered, and incubation continued. The yolk sacs were rinsed, then the tissue and medium processed as described in Section 2.5. Alternate duplicate flasks were opened at each sample time.

## 2.3. Method of Protein Determination

The Folin method of protein assay (Lowry et al., 1951) was used to determine the protein content of tissue samples throughout the project. The tissues were prepared either as soluble digests in 1·0M sodium hydroxide (containing protein at a concentration of about 1mg/ml), or as homogenates in water (containing protein at about 4mg/ml). Duplicate samples of the tissue preparation containing about 0.1mg protein (ie 0.1ml NaOH digest or 20µl homogenate) were made up to a total volume of 0.5ml NaOH, then 0.5ml $H_2O$ added. Standards were prepared over the range 0.0-0.2mg protein using bovine serum albumin. (BSA was dissolved in water at a concentration of 1mg/ml and triplicate sample volumes, of 0-0.2ml, were made up to 0.5ml $H_2O$, then 0.5ml NaOH was added.) Folin A solution (5ml) was added to both tissue samples and standards, then, after at least 20 min, Folin B solution (0.5 ml) was added and the reaction mixture vortexed thoroughly. Colour was allowed to develop for a minimum of 45 min, then the absorbance at 750nm was read against a water blank. The values for the standards were fed into a linear regression program on a calculator from which the line of best fit was determined, and the mg-equivalents of BSA could be calculated for each tissue sample. The correlation coefficient (which can be used as a crude measure of linearity) was always greater than 0.98 in this range of protein concentration.

## 2.4. Methods of Preparation of Tissue and Medium for Assay: Degradable and Non-degradable $^{125}$I-Labelled Substrates

### 2.4a. Non-Degradable: $^{125}$I-PVP

The non-degradable $^{125}$I-labelled substrate used throughout this project was $^{125}$I-polyvinylpyrrolidone ($^{125}$I-PVP) at a final concentration of 2-4µg/ml (depending on the batch). Each rinsed yolk

sac was digested in a total volume of 5ml sodium hydroxide (1M) until totally solubilized. (This usually took 1h at 37°C.) Duplicate 1ml samples of the digest were dispensed into disposable 3ml tubes and assayed for radioactivity in a crystal scintillation gamma counter. Samples of incubation medium (2 x 1ml) were assayed for radioactivity in the same counter. The yolk-sac digest was assayed for protein content as described in Section 2.3.

2.4b. **Degradable Substrate: $^{125}I\text{-}BSA_{fd}$**

$^{125}I\text{-}BSA_{fd}$ was used at a concentration of approximately 1µg/ml. The yolk-sac tissue was treated as described in Section 2.4a. The medium samples contained both macromolecular $^{125}I\text{-}BSA_{fd}$ and $^{125}I$-labelled degradation products of $^{125}I\text{-}BSA_{fd}$ that had been released from the yolk sac. The total radioactivity of the sample (volume 1ml) was measured, then the intact $^{125}I\text{-}BSA_{fd}$ precipitated by the addition of 0.5ml trichloroacetic acid (TCA), 20% w/v, (for medium containing 10% serum), or 0.1ml serum followed by 0.5ml 20% TCA (for serum-free medium). The resulting precipitate was spun down (1000g, 20 min), then the soluble fraction decanted into another tube and assayed for radioactivity. The observed counts in the acid-soluble fraction were corrected for the change in counting volume (which increased from 1.0 to 1.5ml) and for occlusion of acid-soluble material in the pelleted precipitate as described below.

**Determination of Correction Factor:** A solution of $^{125}I$-Tyrosine (labelled by essentially the same method as described for $^{125}I\text{-}BSA_{fd}$ in Section 2.1.) was treated as described above. The carrier protein precipitate was spun down and the TCA-soluble fraction decanted into fresh counting vials and assayed for radioactivity. (This fraction should contain all the radioactivity originally present, except for any which became trapped within the pellet.) The correction factor

was calculated as follows:-

$$\text{Correction factor} = \frac{\text{Total counts (corrected for background) in 1ml solution of } ^{125}\text{I-Tyr}}{\text{TCA-soluble counts (corrected for background)}}$$

## 2.5. Methods of Preparation of Tissue and Medium for Assay: $^{3}H$ - or $^{14}C$-Labelled Substrates

The substrates used were $^{3}H$-inulin, $^{14}C$-sucrose, and $^{3}H$-leupeptin.

Medium samples (0.5ml) were placed in scintillation vial inserts (total capacity 5ml) and 4ml of scintillation fluid added. The contents of the inserts were mixed thoroughly and allowed to stand overnight before assaying for radioactivity in a liquid scintillation counter. Medium 199 contains phenol red which gives it a pink colour. This caused colour quenching of the observed counts in each sample. The counting efficiency of medium relative to distilled water was therefore found, by adding a "spike" (10µl, more than 2000c.p.m.) of tritiated water (for experiments using $^{3}H$-labelled substrates) or $^{14}C$-sucrose (for those using $^{14}C$-sucrose as a substrate) to samples (0.5ml) of distilled water and fresh medium. Scintillation fluid (4ml) was added and the tubes counted at the same time as the experimental samples. The relative counting efficiency was calculated as follows:-

$$\text{Relative counting efficiency of medium with respect to water (\%)} = \frac{\text{Counts (cpm corrected for background) observed in medium}}{\text{Counts (cpm corrected for background) observed in water}} \times 100$$

The observed counts in incubation medium were then corrected for quenching by multiplying by a quench correction factor (ie 100/relative counting efficiency, %).

Several different methods of solubilizing yolk-sac tissue for counting were tested for reproducibility and to minimize quenching. These are described in Appendix 3. The most commonly used method was that of homogenization in a ground-glass homogenizer, which gave good tissue disruption. The relative counting efficiency of samples prepared by this method was determined using an homogenate of un-incubated tissue spiked with $^{3}H_2O$ or $^{14}C$-sucrose. The counting efficiency was reproducible both between samples and stable over prolonged periods of time (when samples were re-counted up to 9 days after addition to scintillation fluid). The counts observed in the experimental yolk-sac samples were corrected for quenching as described above.

## 2.6. Uptake of Non-Radiolabelled Leupeptin

For these experiments, it was necessary to sample a larger quantity of tissue at each time-point in order to obtain sufficient leupeptin from the tissue to analyse. A modification of the reduced volume method (see Section 2.2b) was therefore used. Since the leupeptin was not labelled, it was possible to introduce a control by measuring the uptake of $^{125}I$-PVP into the same yolk sacs as used to monitor leupeptin accumulation.

After dissection, yolk sacs were placed in flasks containing medium 199 (5.6ml, 3 yolk sacs per flask) and allowed to equilibrate for at least 10 min. A solution of leupeptin in distilled water (0.7ml, generally 1mg/ml, except where the effect of different concentrations of leupeptin were studied) and of $^{125}I$-PVP in medium (0.7ml,20-40µg/ml) were then added to each flask to initiate uptake.

In some experiments, a sample of medium (50µl) was immediately removed for leupeptin assay, to find out whether depletion of leupeptin from the medium occurred during incubation. At intervals between 1 and 8h, all three yolk sacs were removed from a flask, together with samples of medium (3 x 1ml). The yolk sacs were rinsed (3 x 2 min) in ice cold 1% saline then either stored at -20°C or immediately prepared for assay of leupeptin and radioactivity content (see Sections 4.2.4, and 5.2.3). One sample of medium was stored at -20°C for assay of leupeptin content, the remaining two samples were counted for $^{125}$I-radioactivity.

## 2.7. Quantitation of Uptake

The quantity of substrate accumulated within yolk sacs by a given time was expressed as the volume of incubation medium (ul) whose contained substrate was captured by a unit quanity (mg) of yolk-sac tissue (see Williams *et al.*, 1975a,b). This method of expression allowed direct comparisons of results obtained from different yolk sacs incubated on different days and with different substrates. The value is independent of the quantity of yolk-sac tissue and method of quantitation used for the substrate (eg beta or gamma radioactivity counting for labelled substrates or assays to detect the mass of inhibitory leupeptin).

For substrates that are neither degraded within nor released from the tissue, the amount of substrate within the tissue at a given time is equal to the total uptake. However if a substrate is released from the tissue (either intact or following degradation), the amount of substrate within the tissue at a given time is not equal to the amount that entered the tissue over that period. Provided the amount of intact/degraded substrate released over a particular period can be measured, the total uptake can be calculated. However, if

quantitation of the amount released is not possible, only the rate of tissue-accumulation (rather than uptake) can be monitored.

The rate of uptake (not accumulation) is termed the Endocytic Index (E.I.), and has the units µl incubation medium per mg yolk-sac protein per hour (µl/mg yolk-sac protein/h). When uptake is substantially linear with time the line of best fit through a plot of uptake values (µl/mg) against time (h) can be found using a linear regression calculator program. The gradient (which is equal to the E.I.), and correlation coefficient (which was used as a crude measure of the degree of scatter of the points) of the line of best fit could both be calculated using this program.

Because the E.I. could only be meaningfully calculated from uptake plots which were linear, all plots were examined by eye to ensure that there was no curvature in the time-course of uptake.

2.7a. **$^{125}$I-Labelled Non-Degradable Substrate: $^{125}$I-PVP** Because this substrate was not degraded or released from yolk-sac tissue (Williams et al., 1975a) the rate of uptake could be calculated directly from the amount of radioactivity within the tissue, as follows:-

$$U = \frac{Y}{M \times P}$$

Where:- U = Uptake (µl/mg yolk-sac protein)

Y = Total radioactivity in the yolk sac (c.p.m., corrected for background)

M = Total radioactivity per µl incubation medium (c.p.m., corrected for background)

P = Protein content of the yolk sac (mg equivalents of BSA)

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The rate of uptake (not accumulation) is termed the Endocytic Index (E.I.), and has the units µl incubation medium per mg yolk-sac protein per hour (µl/mg yolk-sac protein/h). When uptake is substantially linear with time the line of best fit through a plot of uptake values (µl/mg) against time (h) can be found using a linear regression calculator program. The gradient (which is equal to the E.I.), and correlation coefficient (which was used as a crude measure of the degree of scatter of the points) of the line of best fit could both be calculated using this program.

Because the E.I. could only be meaningfully calculated from uptake plots which were linear, all plots were examined by eye to ensure that there was no curvature in the time-course of uptake.

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$$U = \frac{Y}{M \times P}$$

Where:- U = Uptake (µl/mg yolk-sac protein)

Y = Total radioactivity in the yolk sac (c.p.m., corrected for background)

M = Total radioactivity per µl incubation medium (c.p.m., corrected for background)

P = Protein content of the yolk sac (mg equivalents of BSA)

The computer program given in Appendix 1.1 was routinely used to calculate the uptake of $^{125}$I-PVP.

2.7b. **$^{125}$I-Labelled Degradable Substrate: $^{125}$I-BSA$_{fd}$** The total amount of a $^{125}$I-labelled protein taken up is not equivalent to the amount present within the tissue at any one time because the protein is degraded within the tissue to low molecular weight $^{125}$I-labelled degradation products, mostly $^{125}$I-Tyr (see Williams et al., 1975b), which are released back into the medium. However, it is possible to quantitate the amount of TCA-soluble degradation products in the medium (see Section 2.4b), therefore this quantity can be added to the amount of substrate in the yolk sac, to calculate the total uptake at each time-point.

The rate of uptake of $^{125}$I-BSA$_{fd}$ is very high due to its adsorption onto the plasma membrane; this results in significant depletion of substrate from the medium during the incubation period. The mean quantity of substrate present in the medium during each incubation period was therefore used to calculate each value of uptake over a given interval.

Uptake was calculated as follows:-

$$U = \frac{Y + 10\,(S - F)}{M' \times P} \times 10^3$$

Where:- U = Total uptake (µl of incubation medium/mg yolk-sac protein).

Y = Total radioactivity in the yolk-sac (c.p.m., corrected for background)

S = TCA-soluble radioactivity per ml incubation medium at the end of the incubation period (c.p.m., corrected for background and for counting geometry and occlusion, see Section 2.4b).

F = Quantity of TCA-soluble radioactivity per ml of medium that had been incubated at 37°C in the absence of a yolk sac (corrected for background, counting geometry, and occlusion)

M' = Mean quantity of TCA-insoluble radioactivity per ml incubation medium over the particular incubation period.

P = Protein content of yolk sac (mg).

F allows TCA-soluble material (eg $^{125}$I-iodide or $^{125}$I-tyrosine), either present in the $^{125}$I-BSA$_{fd}$ stock solution at the start of the experiment or generated spontaneously from the $^{125}$I-BSA$_{fd}$ during the incubation, to be distinguished from that generated by yolk sac-associated degradation. The value M' was calculated from the total radioactivity observed in the medium sample and the TCA-soluble

Uptake was calculated as follows:-

$$U = \frac{Y + 10\,(S - F)}{M' \times P} \times 10^3$$

Where:- U = Total uptake (µl of incubation medium/mg yolk-sac protein).

Y = Total radioactivity in the yolk-sac (c.p.m., corrected for background)

S = TCA-soluble radioactivity per ml incubation medium at the end of the incubation period (c.p.m., corrected for background and for counting geometry and occlusion, see Section 2.4b).

F = Quantity of TCA-soluble radioactivity per ml of medium that had been incubated at 37°C in the absence of a yolk sac (corrected for background, counting geometry, and occlusion)

M' = Mean quantity of TCA-insoluble radioactivity per ml incubation medium over the particular incubation period.

P = Protein content of yolk sac (mg).

F allows TCA-soluble material (eg $^{125}$I-iodide or $^{125}$I-tyrosine), either present in the $^{125}$I-BSA$_{fd}$ stock solution at the start of the experiment or generated spontaneously from the $^{125}$I-BSA$_{fd}$ during the incubation, to be distinguished from that generated by yolk sac-associated degradation. The value M' was calculated from the total radioactivity observed in the medium sample and the TCA-soluble

radioactivity in that sample as follows:-

$$M' = [M-(S+F)] + \frac{S-F}{2}$$

Where M' and F are as defined above, and M is the total radioactivity observed in the medium sample (c.p.m., corrected for background).

The computer program detailed in Appendix 1.2 was routinely used to calculate the uptake values of $^{125}I\text{-}BSA_{fd}$.

2.7c **$^{14}C$- and $^{3}H$-Labelled Substrates** For these substrates, the radioactivity detected in the yolk sacs and medium could not be compared directly. Beta radioactivity is measured by mixing scintillator with the sample to be counted. Often the sample itself will interfere with the process of energy-transfer between the beta emissions and scintillator, giving rise to quenching. The amount of quenching that occurs depends on factors such as the intensity of colour and protein content of the sample, and the degree of mixing between sample and scintillation fluid. Medium 199 (which is coloured) and solubilized yolk sacs (which contain protein) each produce a different amount of quenching. In order to compare the radioactivity content of the medium with that of yolk sacs to calculate uptake, it was necessary to 'normalize' the observed counts to correct for the different counting efficiency of each. The observed count from a "spike" of radioactivity added to a sample of medium or yolk-sac solution was compared with that from an identical "spike" added to water to find the relative counting efficiency, as described in Section 2.5. The observed counts in samples of medium and yolk sacs derived from uptake experiments could then be

normalized by multiplying by the appropriate relative counting efficiency correction factor. The normalized counts could be directly compared in the numerator and denominator of the 'uptake' equation. Uptake was therefore calculated as follows:-

$$U = \frac{Y \times c.f._{(yolk\text{-}sac)} \times V_{(yolk\text{-}sac)}}{M \times V_{(medium)} \times c.f._{(medium)} \times P}$$

Where:-

$U$ = Uptake (µl/mg)

$Y$ = Yolk-sac associated radioactivity (c.p.m. per yolk sac sample)

$c.f._{(yolk\ sac)}$ = Correction factor for relative counting efficiency of the yolk-sac sample

$V_{(yolk\ sac)}$ = Constant to convert normalized c.p.m. in sample volume assayed, to total normalized c.p.m. for whole yolk sac.

$V_{(medium)}$ = Constant to convert normalized c.p.m. in medium sample assayed, to normalized c.p.m. per µl medium

$M$ = Observed c.p.m. in sample of medium assayed.

$c.f._{(medium)}$ = Correction factor for relative counting efficiency of the medium.

### 2.7d. **Non-Radiolabelled Leupeptin**

Detection of non-radiolabelled leupeptin depended on the leupeptin being in an active, inhibitory form. Any inactive molecules or degradation products could not be detected. Calculations were thus limited to determining the amount of active,

tissue-associated leupeptin. [If no inactivation or loss of leupeptin occurred, this tissue-associated leupeptin would be equivalent to the total uptake. If inactivation or loss did occur, total uptake and thus E.I. could not be calculated.] The amount of active tissue-associated leupeptin was calculated in terms of µl incubation medium per mg yolk-sac protein as follows:-

$$\text{Tissue-associated leupeptin } (\mu l/mg) = \frac{\text{Total active leupeptin associated with the yolk-sac } (\mu g)}{\text{Active leupeptin in medium } (\mu g/ul) \times \text{mg yolk-sac protein}}$$

# CHAPTER 3

# $^{125}$I-BSA$_{fd}$-PROBE METHOD OF LEUPEPTIN DETECTION WITHIN LYSOSOMES IN SITU

## 3.1 INTRODUCTION

Many studies have been made of the effects of leupeptin as an inhibitor of proteolysis within intact cells. However, little has been established concerning the effects of time of exposure to leupeptin and of the concentration of leupeptin in the extracellular fluid on the results of such experiments.

Seglen et al. (1979) reported that, in hepatocytes, the inclusion of a 20min pre-incubation period with leupeptin (120µg/ml) had no effect on results relative to equivalent experiments with no pre-incubation. Similarly, Dean (1979) detected no increase in the degree of inhibition of proteolysis in macrophages over an incubation period of 2-48h in the presence of leupeptin (50µg/ml). Such results are compatible with an immediate and maximal effect of leupeptin on exposure of cells to this inhibitor. Conversely, Tanaka et al. (1979) found that maximal inhibition of BANA-hydrolase activity in hepatocytes did not occur until after 10h incubation with leupeptin (50µg/ml). Some investigators have included, for poorly defined reasons, a pre-incubation period in their experimental designs. Thus, Knowles et al. (1981) included a 30min pre-incubation period in a study of exogenous protein degradation, and Libby and Goldberg (1978) pre-incubated muscle tissue with leupeptin for 90min before measuring degradation.

No systematic investigation of the effect of pre-incubation conditions (time and leupeptin concentration) on the inhibition of proteolysis has been reported. However, unless it can be demonstrated that the observed degree of inhibition of proteolysis is neither a function of an arbitrarily-chosen leupeptin concentration nor of an arbitrarily chosen pre-incubation time, any observed inhibition of proteolysis will be incapable of unequivocal interpretation.

On exposure of cells to leupeptin, the amount of leupeptin that

enters the cells will determine the degree of inhibition of proteolysis. If the rate of penetration of the inhibitor is high (because cellular membranes are freely permeable and the extracellular concentration is high) the agent may rapidly induce a maximal inhibition of proteolysis, providing leupeptin-sensitive proteinases are involved in the rate-limiting steps of proteolysis. (Leupeptin would be expected to act rapidly on reaching its target enzymes, since the affinity of leupeptin for these enzymes is high, Barrett & Kirschke, 1981.) Conversely, if permeation of leupeptin to its site of action is slow, a lag period may be anticipated as the concentration of leupeptin within the intracellular compartments where proteolysis occurs builds up to a level that induces maximal inhibition.

The work described in this chapter therefore investigated the effects of varying both the leupeptin concentration in the medium and the duration of the pre-incubation period, on the observed rates of proteolysis of an exogenous protein. The protein chosen for this study was formaldehyde-denatured $^{125}$I-labelled BSA because it has been shown to be degraded solely within lysosomes following its association with yolk-sac tissue (Livesey & Williams, 1979). Measuring the degree of inhibition of the degradation of this protein thus gives a relative measure of the quantity of active (inhibitory) leupeptin within lysosomes *in situ*. (Without further knowledge of the permeability properties of the lysosomal membrane towards intact leupeptin, it is impossible to say whether or not this is a concentration that exceeds that present in the cytosol.)

In an attempt to establish whether leupeptin was entering the yolk-sac tissue entirely by pinocytosis, several known inhibitors of pinocytosis were used to try to block the uptake of leupeptin. (Possible effects of leupeptin on pinocytosis had to be investigated

as part of the related series of control experiments.) In other experiments a study was made of the regain of proteolytic capacity by tissue that has previously been exposed to leupeptin.

In summary, the experiments reported in this chapter aimed to establish:-

i) The effects of pre-incubating yolk sacs for different periods of time on the degree of inhibition of proteolysis of $^{125}I\text{-}RSA_{fd}$ induced by a given concentration of leupeptin.

ii) The effect of varying the leupeptin concentration during the pre-incubation period on the resulting degree of inhibition of proteolysis.

iii) The mode of uptake of leupeptin by the tissue, (by attempting to block its uptake using known inhibitors of pinocytosis.)

iv) The time-course of regain of proteolytic capacity by tissues previously exposed to leupeptin.

## 3.2. MATERIALS AND METHODS

### I. MATERIALS

All equipment and chemicals are as described in Chapter 2.

### II. METHODS

#### 3.2.1 General Methods

The incubation of yolk sacs was divided into three different periods, termed the pre-incubation, wash, and degradation phases. During the pre-incubation phase, yolk sacs were incubated in the presence of various concentrations of leupeptin, with or without inhibitors of pinocytosis, for various periods of time. A control, in which no leupeptin was present, was also included. The yolk sacs were then rinsed in fresh medium (wash phase), and re-incubated in medium 199 containing $^{125}$I-BSA$_{fd}$ for the degradation phase, during which period the release of TCA-soluble $^{125}$I-BSA$_{fd}$ degradation products into the medium was monitored. The general experimental details were as follows.

After dissection, yolk sacs were placed in flasks containing substrate-free, gassed medium 199 (3 yolk sacs in 6.3ml of medium per flask, see Section 2.2b.). The pre-incubation phase was initiated by the addition of leupeptin (0.7ml stock solution, 1mg/ml in water, diluted if necessary with medium to give a final concentration in the flask of 25-100 µg/ml) or, for the control, medium 199 (0.7ml).

After pre-incubation for a set time, each group of three yolk sacs was transferred, together, to flasks containing warm gassed medium 199 (approximately 20ml) and rinsed for 2 min. Two further such rinses in fresh medium were made. The yolk sacs were then transferred singly to 3 separate flasks containing warm gassed medium

199 and $^{125}I\text{-}BSA_{fd}$ (approx. 10µg/ml, 10ml). Samples of medium (1ml) were removed from each flask at 15 or 30min intervals over a period of 150min: the volume of medium in the flask was kept constant by replenishing with fresh medium. The amount of TCA-soluble $^{125}I\text{-}BSA_{fd}$ degradation product (mainly $^{125}I$-Tyr; Williams et al., 1975b; Livesey & Williams, 1979) was estimated by adding 0.1ml serum (as a carrier protein) followed by 0.5ml TCA (20% w/v) to each sample to precipitate intact $^{125}I\text{-}BSA_{fd}$. The precipitate was spun down (1000g, 20min) and the soluble fraction transferred to new counting vials for assay of radioactivity. In some experiments leupeptin was also present in the wash and degradation phases. The experiment was performed as above except the concentration of leupeptin was the same as that in the pre-incubation phase throughout the experiment.

### 3.2.2. Calculation and Expression of Results

The total amount (in c.p.m.) of TCA-soluble radioactivity released into the medium after each sample time was calculated as follows:-

$$T_n = \left[ 10\, C_{i(i=n)} \cdot f + \sum_{i=0}^{i=n-1} C_i \cdot f \right] \div P$$

where $T_n$ = Total radioactivity released into the medium (c.p.m.) up to the time of removal of the nth sample (c.p.m. per mg yolk-sac protein)

$P$ = Protein content of the yolk sac (mg)

$C_i$ = Radioactivity per ml medium in the ith sample (c.p.m. corrected for background)

$f$ = Counting geometry correction factor (see Section 2.4b)

Data were routinely processed using the computer program given in Appendix 1.3.

The mean value of $T_n$ for the 3 yolk sacs that underwent identical pre-incubation phases was calculated and the results plotted as a graph of mean c.p.m. released ($T_n$) against degradation phase incubation period. The slope of the graph between 60-150 mins of incubation was determined as a measure of the rate of degradation of $^{125}I\text{-}BSA_{fd}$ by the tissue. The rate observed in leupeptin-treated tissue was expressed as a percentage of that observed in control tissue.

### 3.2.3. Recovery of the Proteolytic Capacity of Tissue after Exposure to Leupeptin

The reversibility of the leupeptin-induced inhibition of $^{125}I\text{-}BSA_{fd}$ degradation was measured by determining the time-course and extent of regain of proteolytic capacity by the tissue. After pre-incubation with leupeptin (100µg/ml for 45mins) the tissues were rinsed in three changes of fresh medium (generally 3 x 4min) then transferred, together, to substrate-free medium 199 (approximately 10ml per flask) for recovery periods of 60-180 min. (A recovery period of 6min was obtained by reducing the wash period to 3 x 2min.)

The yolk sacs were then transferred singly to separate flasks containing medium 199 plus $^{125}I\text{-}BSA_{fd}$ and the degradation phase of the experiment continued as described in Section 3.2.1.

### 3.2.4. Variation of Duration of the Pre-incubation Phase and of the Leupeptin Concentrations

The method was essentially as described in Section 3.2.1. The pre-incubation period was varied from 10-180min, with leupeptin at concentrations of 25, 50 and 100µg/ml medium.

Data were routinely processed using the computer program given in Appendix 1.3.

The mean value of $T_n$ for the 3 yolk sacs that underwent identical pre-incubation phases was calculated and the results plotted as a graph of mean c.p.m. released ($T_n$) against degradation phase incubation period. The slope of the graph between 60-150 mins of incubation was determined as a measure of the rate of degradation of $^{125}I\text{-}BSA_{fd}$ by the tissue. The rate observed in leupeptin-treated tissue was expressed as a percentage of that observed in control tissue.

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The yolk sacs were then transferred singly to separate flasks containing medium 199 plus $^{125}I\text{-}BSA_{fd}$ and the degradation phase of the experiment continued as described in Section 3.2.1.

### 3.2.4. Variation of Duration of the Pre-incubation Phase and of the Leupeptin Concentrations

The method was essentially as described in Section 3.2.1. The pre-incubation period was varied from 10-180min, with leupeptin at concentrations of 25, 50 and 100µg/ml medium.

### 3.2.5. Effect of Inhibitors of Pinocytosis on the Uptake of Leupeptin during Pre-incubation

The inhibitors used in this study were ammonium chloride (20mM) and low temperature (6, 15 and 20°C), as these were shown to be more readily reversible than other conditions that inhibit pinocytosis (see Section 3.3.4). Before addition of leupeptin to initiate the 45min pre-incubation phase, yolk sacs were allowed to equilibrate in either medium 199 containing ammonium chloride (20mM, 6.3ml) or in medium 199 (6.3ml) maintained at 6, 15 or 20°C. Control experiments were carried out in which yolk sacs were exposed to no inhibitor (ie maintained at 37°C in medium 199 alone), leupeptin only (ie no exposure to ammonium chloride or low temperatures), or pinocytic inhibitor only (ie equilibrated with ammonium chloride without leupeptin: no equivalent low temperature treatment was carried out), before transfer to medium containing $^{125}I\text{-}BSA_{fd}$. The rate of production of TCA-soluble material by tissue that had been exposed to both leupeptin and an inhibitor of pinocytosis was expressed as a percentage of that by tissue exposed to the pinocytic inhibitor alone. The rate observed in tissue that had been exposed only to leupeptin was expressed as a percentage of the control rate, for tissue that had been pre-incubated in medium 199 alone.

### 3.2.6. Reversibility and Effectiveness of Inhibitors of Pinocytosis in the Rat Yolk Sac

For the experiments described in Section 3.2.5., it was important that the effects of any inhibitor used to block pinocytosis during the pre-incubation phase were rapidly and fully reversible, so that its effects did not continue during the degradation phase. Continued inhibition of either uptake or degradation of the $^{125}I\text{-}BSA_{fd}$ by the inhibitor of pinocytosis used would lead to

confusion in the interpretation of results. It was also important that any such inhibitor should totally block pinocytosis, since any small residual entry of leupeptin into the tissue by pinocytosis would affect the subsequent rate of $^{125}I\text{-}BSA_{fd}$ degradation and could be misinterpreted as signifying non-pinocytic uptake of some of the leupeptin. The degree of inhibition of pinocytosis and the reversibility of several inhibitors were tested as described below.

3.2.6a **Degree of Inhibition of Pinocytosis** The degree of inhibition of degradation of $^{125}I\text{-}BSA_{fd}$ was taken as a measure of the effectiveness of the putative inhibitors of pinocytosis.

Yolk sacs were pre-incubated separately in the presence of the inhibitor for 10-60min, then $^{125}I\text{-}BSA_{fd}$ was added to the medium. The release of TCA-soluble radioactivity was monitored as described in Section 3.2.1.; the medium used to replenish the sample volumes contained inhibitor at the same concentration as the sample. For some experiments, the Endocytic Index rather than rate of $^{125}I\text{-}BSA_{fd}$ degradation was determined, by assaying medium and yolk-sac tissue at various intervals (as described in Section 2.2a, 2.4b and 2.7b). Inhibitors tested were rotenone ($10^{-5}$M), monensin (50mM), high $K^+$:$Na^+$ concentration (1M solutions of $NaH_2PO_4$ and $K_2HPO_4$ titrated together to pH 7.3, then 0.5ml buffer added to 9.5ml medium), EGTA (5mM and 1mM), fluoride (20 µg/ml), ammonium chloride (20mM), and low temperature (6-20°C). The more suitable inhibitors were further tested for their ability to inhibit uptake of $^{125}I\text{-}PVP$, using the method described in Section 2.2a and 2.4a. The inhibitor was included in the medium in which the yolk sacs were incubated, and tissue was allowed to equilibrate for 30 minutes before addition of $^{125}I\text{-}PVP$.

3.2.6b **Reversibility of Effects of Inhibitors: Regain of Pinocytic and Proteolytic Capacity** Tissue was incubated in the presence of various compounds reported to inhibit pinocytosis in the yolk sac. In most cases the experiments were performed in a similar fashion to that described in Section 3.2.1. except that tissue was pre-incubated for 1 hour in the presence of an inhibitor of pinocytosis, rather than with leupeptin. The tissue was rinsed (x3) in changes of substrate-free medium for up to 30min, to allow time for recovery to occur, then transferred to medium containing $^{125}I\text{-}BSA_{fd}$ and the rate of production of TCA-soluble material monitored as described in Sections 3.2.1. and 3.2.2. [In some experiments, $^{125}I\text{-}BSA_{fd}$ was present at the same time as the inhibitor, since the same tissue was used for investigation of both effectiveness and reversibility of an inhibitor. After monitoring the production of TCA-solubles in the presence of inhibitor as described in Section 3.2.6a the tissue was rinsed in fresh medium as described above then transferred to fresh inhibitor-free medium containing $^{125}I\text{-}BSA_{fd}$.] Controls were treated in the same way as experimental tissue except that no inhibitor was present during any part of the incubation.

Inhibitors tested for reversibility were rotenone ($10^{-5}M$), EGTA (5mM, with or without 5mM $CaCl_2$ supplement in the degradation phase medium), methylamine (20mM), ammonium chloride (20mM), and low temperatures (6,15 and 20°C).

3.2.7. Effect of Pre-incubation with Leupeptin on the Subsequent Rate of Pinocytosis

Yolk sacs were pre-incubated in the presence or absence of leupeptin (100µg/ml) as described in Section 3.2.1. Tissue was then washed in three changes of fresh medium (12 min) and transferred to flasks containing $^{125}I\text{-}PVP$ in medium 199 (approximately 3µg/ml,

10ml). The uptake of $^{125}$I-PVP over a period of 120min was monitored as described in Sections 2.2 and 2.4a, and the Endocytic Indices of leupeptin-treated and control tissues were determined as described in Section 2.7a.

## 3.3 RESULTS

### 3.3.1 Effect of Pre-incubation of Yolk-Sac Tissue with Leupeptin, on the Subsequent Uptake of $^{125}$I-PVP

The Endocytic Index of $^{125}$I-PVP for tissue that had been pre-incubated with leupeptin was determined, to establish whether pinocytosis was affected during the subsequent degradation phase in corresponding experiments with $^{125}$I-BSA$_{fd}$ as substrate.

The E.I. (mean ± standard deviation) of leupeptin-treated and of control tissues was 3.35 ± 0.47 and 3.64 ± 0.40 respectively. There was no marked difference between the two mean E.I.'s, indicating that the pinocytic capacity of the tissue was unaffected by pre-incubation with leupeptin.

### 3.3.2. Recovery of the Proteolytic Capacity of Tissue after Exposure to Leupeptin

**3.3.2a. Effect of Pre-incubation of Tissue with Leupeptin on Subsequent Degradation of $^{125}$I-BSA$_{fd}$** Pre-incubation of tissue with leupeptin greatly affected the ability of the tissue to degrade $^{125}$I-BSA$_{fd}$. Fig. 3.1 shows a typical plot of the time-course of release of TCA-soluble $^{125}$I-BSA$_{fd}$ degradation products from tissue that had been pre-incubated without leupeptin (control), pre-incubated for 1 hour with leupeptin (100µg/ml), or incubated with leupeptin (100µg/ml) throughout the pre-incubation, wash and degradation phases.

In control tissue the TCA-soluble radioactivity was released into the medium at a constant rate, with no lag phase over the initial period. The rate of release of TCA-soluble radioactivity was much greater than that in tissues that had been exposed to leupeptin. Tissue that had been exposed to leupeptin for one hour showed a pattern of release of TCA-solubles similar to that observed for

tissue that had been exposed to leupeptin continuously, although in tissue exposed for 1h the increase in rate after 60 minutes degradation phase was more marked, and release of TCA-solubles was greater than for tissue continuously exposed to leupeptin. The time-course of release of TCA-solubles between 60-150 minutes of degradation phase was approximately linear in all cases; the rate in these linear regions was calculated and the inhibited rate expressed as a percentage of the rate in control yolk sacs to determine the percentage remaining degradative capacity (as described in Section 3.2.2.). Results for several determinations following one hour exposure to leupeptin (100µg/ml) are shown in Table 3.1. The value of c.p.m./mg yolk-sac protein/min varied from day to day according to the specific radioactivity of the $^{125}$I-BSA$_{fd}$ preparation and the gamma-counter efficiency. This variation was normalized by expressing the rate as a percentage of the matched control. The mean percentage remaining degradative capacity for this pre-incubation regime was 33.7%. (The equivalent value for tissues continually exposed to leupeptin was 18.0%.)

A lag in onset of degradation was observed for all tissues that had been exposed to leupeptin. Further investigations into the cause of the observed lag period were made by varying the recovery time between the pre-incubation phase, with leupeptin, and the degradation phase. This also gave an indication of the time course of any recovery of the capacity to degrade $^{125}$I-BSA$_{fd}$ after leupeptin exposure.

3.3.2.b **Effect of Variation of Recovery Periods** The tissue was incubated as described in Section 3.2.3. Pre-incubated tissue was allowed to recover in medium containing neither leupeptin nor $^{125}$I-BSA$_{fd}$ for 6, 12, 60, 120 and 180 minutes, before transfer to medium containing $^{125}$I-BSA$_{fd}$ for the degradation phase. Fig 3.2 shows

the time-course of recovery by the tissue, expressed in terms of the percentage residual degradative capacity. Although this value varied somewhat between experiments there was no overall increase in the capacity of the tissue to degrade $^{125}$I-BSA$_{fd}$ throughout the recovery period.

The effect of the recovery period on the observed delay in onset of release of TCA-solubles is shown in Fig 3.3, which is a typical plot of the quantity of $^{125}$I-BSA$_{fd}$ degraded (c.p.m. released/mg protein) against degradation-phase time. A lag phase of approximately 60min was apparent in all cases, even after 180min recovery. This indicates that the delay in onset of $^{125}$I-BSA$_{fd}$ degradation was not dependent on the period between the pre-incubation and degradation phases but rather on the period of exposure to $^{125}$I-BSA$_{fd}$, and/or to leupeptin.

### 3.3.3. Variation of Pre-incubation Phase Conditions: Effects of Leupeptin Concentration and Exposure Time.

These experiments were conducted in order to obtain some indication of the rate of action (and hence uptake) of leupeptin at various concentrations. The experiments were carried out as described in Section 3.2.1. The final leupeptin concentration was 25, 50 or 100µg/ml and the pre-incubation periods were 10, 20, 30, 45, 60, 120 and 180min. Typical plots of the TCA-solubles released (c.p.m. per mg yolk-sac protein) against degradation-phase time, for 25, 50 and 100µg/ml are shown in Figs 3.4, 3.5 and 3.6 respectively.

The degree of inhibition observed rose with increasing pre-incubation time. For leupeptin at a concentration of 100µg/ml, a maximum inhibition was reached after approximately 60min; no further inhibition was observed after longer pre-incubation times. It was noteworthy that the magnitude of the lag period, described in

the time-course of recovery by the tissue, expressed in terms of the percentage residual degradative capacity. Although this value varied somewhat between experiments there was no overall increase in the capacity of the tissue to degrade $^{125}$I-BSA$_{fd}$ throughout the recovery period.

The effect of the recovery period on the observed delay in onset of release of TCA-solubles is shown in Fig 3.3, which is a typical plot of the quantity of $^{125}$I-BSA$_{fd}$ degraded (c.p.m. released/mg protein) against degradation-phase time. A lag phase of approximately 60min was apparent in all cases, even after 180min recovery. This indicates that the delay in onset of $^{125}$I-BSA$_{fd}$ degradation was not dependent on the period between the pre-incubation and degradation phases but rather on the period of exposure to $^{125}$I-BSA$_{fd}$, and/or to leupeptin.

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The degree of inhibition observed rose with increasing pre-incubation time. For leupeptin at a concentration of 100µg/ml, a maximum inhibition was reached after approximately 60min; no further inhibition was observed after longer pre-incubation times. It was noteworthy that the magnitude of the lag period, described in

Section 3.3.2, seemed to depend on the pre-incubation period with leupeptin (being less apparent for short pre-incubation periods). Figs. 3.7 a,b,c,d,e show a comparison of the rate of degradation of $^{125}$I-BSA$_{fd}$, in matched experiments, in tissue exposed to 50 and 100µg/ml leupeptin for various periods. Initially, the degree of inhibition was greater in those tissues exposed to the higher concentration of leupeptin, however after 180 minutes the degree of inhibition was the same for both concentrations. This degree of inhibition was the maximum observed for leupeptin at 100µg/ml. Fig. 3.8 shows this effect more clearly, by expressing the rate of degradation as the percentage residual degradative capacity. The residual degradative capacity in tissue exposed to leupeptin at 100µg/ml decreased fairly rapidly (approximately 15% inhibition was apparent after only 10min) up to 60min exposure time, after which it was more or less constant up to 240min. For leupeptin at 50µg/ml, the residual degradative capacity decreased more slowly over 10-180min pre-incubation time, but eventually reached the same maximal inhibition observed with leupeptin at 100µg/ml. Leupeptin at a concentration of 25µg/ml gave the slowest decrease in residual proteolytic capacity, and the maximum degree of inhibition reached was never as great as for concentrations of 50 or 100µg/ml.

The maximum inhibition achieved was never total; approximately 20% of the degradation of $^{125}$I-BSA$_{fd}$ continued to occur.

3.3.4 Effectiveness and Reversibility of Inhibitors of Pinocytosis

3.3.4a **Effectiveness of Inhibitors** The rate of degradation of $^{125}$I-BSA$_{fd}$ in the presence of different inhibitors of pinocytosis was monitored in an initial assessment of the degree of inhibition of pinocytosis and/or degradation. The results are shown in Table 3.2a. Sodium fluoride (20µg/ml) and EGTA (1mM) were found to have no effect on the rate of degradation of $^{125}$I-BSA$_{fd}$, indicating that uptake was not inhibited, and rotenone ($10^{-5}$M) gave only partial inhibition. The most effective inhibitors were ammonium chloride at 20mM (lower concentrations gave partial inhibition) and low temperature (6°C), (both of which inhibited virtually all release of TCA-soluble material), and methylamine, (which inhibited the majority of TCA-soluble release). Monensin (50mM) and high $K^+$:$Na^+$ concentration were tested for effectiveness by monitoring the Endocytic Index of $^{125}$I-BSA$_{fd}$ in treated tissue, rather than the rate of release of TCA-soluble material alone. Table 3.2b shows these results. Monensin gave approximatly 75% inhibition of uptake and degradation, and a high $K^+$:$Na^+$ concentration gave almost total inhibition. For monensin, the total amount of radioactivity associated with the tissue remained fairly high, suggesting that pinocytosis occurred to some extent, but degradation was inhibited. A high $K^+$:$Na^+$ concentration decreased the amount of radioactivity within the yolk-sac, suggesting that pinocytosis was the inhibited step.

3.3.4b **Reversibility of Effects of Inhibitors** Tissue was incubated in the presence of the inhibitor for 1h then rinsed and either transferred immediately to flasks containing $^{125}$I-BSA$_{fd}$ or allowed to recover for up to 30min in the final wash flask, before transfer to $^{125}$I-BSA$_{fd}$-containing flasks. The rate of release of

TCA-solubles radioactivity was then monitored to determine whether full proteolytic capacity of the tissue towards $^{125}I\text{-}BSA_{fd}$ was recovered. This required regain of both pinocytic and proteolyic activity, since pinocytosis is normally rate-limiting in the process. The results are given in Table 3.3. The values shown are typical for each inhibitor. (It was not possible to take mean values of the rate of release of TCA-solubles (c.p.m./mg/min) because of the variation in this value from day to day.)

Methylamine and rotenone were shown to be not reversible, althouqh some recovery did occur after 30min. EGTA was also not reversible, particularly so if $Ca^{2+}$ was included in the medium in an attempt to counter-act the sequestration of $Ca^{2+}$ions. Exposure of the tissue to temperatures below 37°C appeared to enhance the subsequent rate of degradation of $^{125}I\text{-}BSA_{fd}$, lower temperatures producing greater enhancement. This experiment did not distinquish whether this was caused by an increase in the rate of pinocytosis or of proteolysis. (A similar effect was observed with ethanol-exposed yolk sacs; the rate of uptake of $^{125}I$-PVP was increased, suggesting pinocytosis rather than proteolysis was stimulated. G.Steventon, unpublished data.) The effects of ammonium chloride were readily reversible, even after only nine minutes rinse/recovery period. The degree of recovery was somewhat variable, a mean percentage recovery value for all such experiments (with different wash times) was 90.4%, standard deviation 25.2

Since ammonium chloride gave a high degree of inhibition of release of TCA-solubles together with a rapid and comparatively complete recovery it was considered suitable for use in attempts to inhibit any pinocytic uptake of leupeptin. However, ammonium chloride is a potent inhibitor of proteolysis, therefore it was necessary to check that the inhibition of release of TCA-solubles was

caused by an inhibition of pinocytosis, rather than just proteolysis. Uptake of $^{125}$I-PVP in the presence of ammonium chloride (20mM) was therefore monitored as described in Section 3.2.6a. The mean E.I. was 0.16, whereas that for control tissue was 1.6. Pinocytosis was thus inhibited 90% by ammonium chloride at this concentration.

### 3.3.5 Effect of Inhibitors of Pinocytosis on Entry of Leupeptin into Yolk-Sac Tissue

The method used in these experiments is described in Section 3.2.5. As shown in Section 3.3.4a,b., ammonium chloride and low temperature are potent, rapidly-reversible inhibitors of pinocytosis, therefore their inclusion in the pre-incubation phase with leupeptin should prevent any pinocytic uptake of leupeptin. Their effects should then be reversed during the standard wash phase so that pinocytosis and degradation of $^{125}$I-BSA$_{fd}$ would occur normally. Typical plots of release of TCA-solubles (c.p.m./mg yolk-sac protein) against degradation phase time, after pre-incubation with leupeptin in the presence of ammonium chloride (20mM), are shown in Figs 3.9 a,b,c,and d. The same data are also expressed as a percentage of the matched control in Table 3.4. The figures clearly show that release of TCA-soluble radioactivity was inhibited in leupeptin-treated tissue, whether or not $NH_4Cl$ was present during the leupeptin exposure period. Hence, it would appear ammonium chloride did not fully prevent entry of some leupeptin into the lysosomes, suggesting that uptake of leupeptin was not entirely dependent on pinocytosis. However, residual proteolytic activity within the lysosomes was greater in tissue that had been incubated in the presence of leupeptin and ammonium chloride, than in that exposed to leupeptin alone. This indicates that the amount of leupeptin present within the lysosomes was greater when ammonium chloride was

absent. This observation suggests either a reduced uptake of leupeptin in the presence of the inhibitor or an increased rate of loss of leupeptin from lysosomes containing ammonium chloride.

The results obtained using low temperature as an inhibitor of pinocytosis in the pre-incubation phase are shown in Fig.3.10. The rate of degradation of $^{125}I\text{-}BSA_{fd}$ after treatment with leupeptin at a low temperature was calculated as a percentage of that measured in yolk-sacs that had been pre-incubated at 37°C in the absence of inhibitor. Results given in Section 3.3.4b suggested that $^{125}I\text{-}BSA_{fd}$ degradation (at 37°C) was enhanced after pre-incubation at a lower temperature. This suggestion is further supported by the results in Fig. 3.10, since in many cases the rate of degradation of $^{125}I\text{-}BSA_{fd}$ was greater in low temperature/leupeptin-treated tissue than in the controls maintained at 37°C throughout. These data do not allow clear conclusions to be drawn as to whether leupeptin uptake was abolished at low temperatures. However, the amount of leupeptin within the tissue (as determined by the residual degradative capacity) did appear to be temperature dependent, since the degree of inhibition given by leupeptin increased as the temperature of pre-incubation increased from 6 - 20°C, even though at these temperatures the control rate (37°C) was less than or equal to the inhibited rate.

## 3.4. DISCUSSION

### 3.4.1. General features of $^{125}I\text{-}BSA_{fd}$ degradation as a probe for leupeptin

The method of estimating the amount of leupeptin associated with yolk-sac tissue described in this chapter has a number of advantages:-

i) The method relies on inhibition of lysosomal function in intact, living tissue and therefore relates to normal lysosomes *in situ* (rather than to, for example, diluted lysosomal extract under arbitary conditions).

ii) The method circumvents any problems that might be associated with obtaining leupeptin from tissue in an extract suitable for the measurement of leupeptin content.

iii) The substrate was a protein, rather than a low molecular mass chromogenic or fluorogenic substrate, hence any observed inhibition is likely to be more representative of the effects of leupeptin on proteolysis in general.

iv) The assay detects intralysosomal leupeptin only. Any leupeptin bound to the external cell surface, rather than being internalized, would not be detected. (In contrast, such non-internalized substrate may be included in the value for total uptake when using other methods to measure uptake.)

v) The assay was found to be sensitive to small amounts of leupeptin. (For example a decrease in rate of degradation of $^{125}I\text{-}BSA_{fd}$ could be detected after only ten minutes exposure of tissue to leupeptin at a concentration of 100µg/ml.)

The success of the method depended on certain factors described below.

i) The protein substrate must be degraded intracellularly. If any

degradation occurred at the surface of the plasma membrane, or by free proteinases released into the bulk medium, interpretation of the observations would be difficult. However it has been established that degradation of $^{125}$I-BSA$_{fd}$ by yolk sacs is entirely intralysosomal (Livesey & Williams, 1979), so any observed effects of leupeptin must relate to an inhibition of lysosomal function.

ii) Leupeptin must not affect the rate of pinocytosis by the tissue. Any inhibition of pinocytosis caused by leupeptin would affect the rate of degradation of $^{125}$I-BSA$_{fd}$, since pinocytic capture of a protein is the rate-limiting step in its degradation within yolk sacs (Williams et al., 1975b). Thus, in the assay, any such inhibition of pinocytosis would cause an inhibition of degradation. This in turn would be mistakenly equated with inhibition of lysosomal enzymes.

iii) For those experiments in which leupeptin was present in the pre-incubation phase only, the inhibition of lysosomal enzymes caused by leupeptin must not be rapidly reversible, otherwise little or no effect would be present during degradation phase. If the effects of leupeptin are rapidly reversible and/or some or all of the leupeptin is lost from the tissue during the wash phase, the amount of leupeptin that enters the tissue during the pre-incubation phase could not be assessed by measuring inhibition of degradation of $^{125}$I-BSA$_{fd}$ during the leupeptin-free degradation phase. To detect different amounts of tissue-associated leupeptin, either the duration of the period of inhibition or the degree of inhibition (or both) must differ during the degradation phase after exposure of the tissue to different incubation regimes with leupeptin.

The method was not without disadvantages. The main disadvantage of the method was that the absolute quantity of leupeptin within the lysosomes could not be calculated, since the relationship between intralysosomal concentration of intact leupeptin and the rate of degradation of $^{125}I\text{-}BSA_{fd}$ was not known. Another disadvantage was that only leupeptin within the lysosomal system was detected, so the leupeptin content of the entire tissue could not be assessed. The relative proportion of leupeptin in lysosomal or non-lysosomal compartments would depend on the permeability of the plasma and lysosomal membranes toward leupeptin, therefore measurements of the lysosomal content of leupeptin may not reflect the total tissue content of leupeptin.

Overall, this method of detecting leupeptin permits a semi-quantitative estimate of the amount of active leupeptin within the lysosomal system of intact tissue to be made.

### 3.4.2. Discussion of Results Obtained Using This Method

The main findings of this chapter are interesting when compared with the literature on the degree of inhibition of degradation caused by leupeptin for different classes of proteins (eg short-lived, long-lived, abnormal, endogenous or exogenous) in different cell types (see reviews by Seglen, 1983; Dean, 1980). Experiments show that the degree of inhibition varies a great deal, from no inhibition of degradation of abnormal endogenous proteins in hepatocytes (Neff *et al.*, 1979) to 80% inhibition of degradation of exogenous $^{125}I\text{-}BSA_{fd}$ in the yolk sac (Knowles *et al.*, 1981). The differences were assumed to indicate differences in the contribution of leupeptin-sensitive thiol proteases towards degradation of proteins of different half-lives and in different cell types. However care should be exercised when comparing these results, since not only did

the type of cell and class of protein studied differ between experiments, but also the concentration of leupeptin used, the pre-incubation period with leupeptin, and the period over which degradation was studied. Changes in any of these factors could lead to differences in the observed degree of leupeptin-induced inhibition. Indeed, results described in this chapter show that the degree of inhibition of degradation of $^{125}$I-BSA$_{fd}$ in rat yolk sacs was dependent on both the period of exposure to, and concentration of, leupeptin during the pre-incubation phase.

Onset of inhibition occurred after only ten minutes exposure to leupeptin, suggesting fairly rapid entry of leupeptin into the tissue. The degree of inhibition observed rose progressively with time, maximal inhibition was not achieved until after one hour exposure to leupeptin (100 µg/ml). Lower concentrations of leupeptin in the pre-incubation medium required longer periods of exposure to achieve the same degree of inhibition. These observations indicate that, for a given leupeptin concentration, the amount of leupeptin within the lysosomal system increased gradually with increasing time of exposure to leupeptin, at least up to the time when maximum inhibition was observed.

Several explanations could be put forward to account for the constant maximum degree of inhibition of $^{125}$I-BSA$_{fd}$ degradation, and why this was not equal to 100%. The most likely is that the remaining 20% of degradation was mediated by leupeptin-insensitive enzymes in the lysosome, such as cathepsins D and possibly H (which is not very sensitive to leupeptin). The relative contribution of various lysosomal proteinases towards the degradation of $^{125}$I-BSA$_{fd}$ *in vivo* is unknown, though purified cathepsins B, D, H, and L are all capable of hydrolysing $^{125}$I-BSA$_{fd}$ (Kooistra *et al.*, 1982). Any degradation mediated by leupeptin-insensitive enzyme(s) would still

take place if leupeptin-sensitive enzymes were fully inhibited, and may even occur to a greater extent when the leupeptin-sensitive pathways become blocked (Tanaka et al., 1981). Further increases in the leupeptin content of the lysosomes would not lead to any further inhibition of degradation of $^{125}I\text{-}BSA_{fd}$.

Other explanations can be dismissed on consideration of experimental evidence. $^{125}I\text{-}BSA_{fd}$ has been shown to be degraded entirely within lysosomes (Livesey and Williams, 1979) so the residual degradation could not be attributed to a leupeptin-insensitive, non-lysosomal pathway. Degradation of $^{125}I\text{-}BSA_{fd}$ via a population of lysosomes that did not contain leupeptin was considered unlikely. (The only source of such lysosomes would be a new population of primary lysosomes, and these would only remain leupeptin-free if the lysosomal membrane is not permeable to leupeptin and if they did not fuse with existing lysosomes that contained leupeptin. Also, turnover of lysosomes during the experimental period would not be expected to occur to any great extent; lysosomal components are reported to have a half-life of about 30h (Dean, 1977).

An alternative explanation is that leupeptin itself was degraded within the lysosomes to reach a steady-state concentration (when the rate of degradation was equal to the rate of uptake), this concentration giving rise to an 80% inhibition of degradation of $^{125}I\text{-}BSA_{fd}$. However, if leupeptin is inactivated, the tissue could be expected to regain its proteolytic activity after exposure to leupeptin; no such recovery was observed, even after a 3h recovery period.

The rate of degradation of $^{125}I\text{-}BSA_{fd}$ in the continuous presence of leupeptin was lower than that in tissues that had only been exposed to the inhibitor for one hour. This may have been due to

partial recovery of the tissue on removal of leupeptin. Alternatively, the increased leupeptin exposure time of the continuously-exposed tissue may have led to the increased inhibition observed (indeed degrees of inhibition similar to that observed for continuous presence, ie 18%, were observed in some of the experiments in which tissue was pre-incubated for 180 minutes with leupeptin 100µg ml, then re-incubated over the degradation phase without leupeptin).

Leupeptin is a competitive inhibitor (Umezawa and Aoyagi, 1977) and is therefore theoretically reversible. Previous studies using rat yolk sacs demonstrated that inhibition of exogenous (though not endogenous) protein degradation by leupeptin was indeed partially reversible after a two hour recovery period (Knowles et al., 1981). However, Seglen et al. (1979) found leupeptin to be irreversible in practice when working with hepatocytes, and Kirschke et al. (1977) found that leupeptin acted as a pseudo-irreversible inhibitor towards purified cathepsin L. In the experiments reported in this chapter, a decreased rate of degradation of $^{125}$I-BSA$_{fd}$ was observed after incubation with leupeptin, even though leupeptin was not present during the period when the rate of degradation was measured. Indeed the rate of degradation was inhibited to a similar extent in tissue that had been allowed to recover in medium free from both leupeptin and $^{125}$I-BSA$_{fd}$ for up to 180 minutes before monitoring the degradation. This continued inhibition must have been caused by a continuous presence of leupeptin within the lysosomes (at least up to the maximum period monitored, which was 5.5 hours).

There are several mechanisms by which leupeptin might become trapped in lysosomes. First, if lysosomal and plasma membranes were impermeable towards leupeptin, the only route of entry of leupeptin into the tissue would be via pinocytosis, leading to delivery to the

partial recovery of the tissue on removal of leupeptin. Alternatively, the increased leupeptin exposure time of the continuously-exposed tissue may have led to the increased inhibition observed (indeed degrees of inhibition similar to that observed for continuous presence, ie 18%, were observed in some of the experiments in which tissue was pre-incubated for 180 minutes with leupeptin 100µg ml, then re-incubated over the degradation phase without leupeptin).

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There are several mechanisms by which leupeptin might become trapped in lysosomes. First, if lysosomal and plasma membranes were impermeable towards leupeptin, the only route of entry of leupeptin into the tissue would be via pinocytosis, leading to delivery to the

lysosomal system. Leupeptin would then remain trapped within lysosomes and loss could only occur via exocytosis or degradation of leupeptin. Second, even if the membranes were permeable to leupeptin, the leupeptin may still become trapped within lysosomes by virtue of its binding to lysosomal enzymes. (Leupeptin is a tight-binding inhibitor with a fairly low $K_i$ for lysosomal enzymes; $K_i$ (apparent) values of $7 \times 10^{-9}$M for cathepsin B, approximately $3.7 \times 10^{-8}$M for cathepsin L, but only about $5 \times 10^{-6}$M for cathepsin H, were reported by Barrett & Kirschke, 1981), hence it may remain bound to the enzymes even if the concentration of leupeptin in the tissue was low, giving rise to the continued inhibition. Third, the leupeptin molecule has a positively charged arginal group which may possibly act in a lysosomotropic manner. (Lysosomotropic agents are normally molecules that are uncharged at neutral pH, and therefore able to permeate the lysosomal membranes freely, but become protonated within the lower pH of lysosomes so cannot pass out through the membrane. The $pK_a$ of the guanidinyl group of arginine is 12.48 therefore it would be expected to be charged even at neutral pH, which may render the molecule resistant to membrane permeation. However, the molecule can cyclize (see Fig.1.1), and the cyclized form may have an altered $pK_a$ and behave as a conventional weak base.)

The lack of recovery of proteolysis also suggests either that no new population of primary lysosomes was synthesised during the experimental period (as suspected from previous results) or that such lysosomes were rapidly inhibited to the same extend as pre-existing lysosomes by excess leupeptin within the tissue.

The release of TCA-soluble radioactivity in control tissue (ie that which had not been exposed to leupeptin) was linear over the entire degradation phase. However, tissue that had been exposed to leupeptin showed a lag phase of up to 60 minutes during which

degradation was either very slow or did not occur. After this period, the degradation of $^{125}I\text{-}BSA_{fd}$ increased to a constant rate which was used to calculate the degree of inhibition of proteolysis. The length of the lag period seemed to depend on both the concentration of leupeptin to which the tissue had been exposed and the duration of such exposure. Shorter lag periods were observed in tissue that has been exposed to leupeptin for a shorter period of time and/or to lower concentration of leupeptin.

Several possible causes may be put forward to explain the lag phase; some of these could be ruled out on the basis of further investigations. A study of the time-course of recovery of proteolysis in the yolk sac showed that lag periods were observed in tissue that had been allowed to recover for 180 minutes, and were of a similar duration to those observed in tissue that had not undergone a recovery period. Hence a rapid partial loss of leupeptin over the first 60min after pre-incubation could be rejected as an explanation of the lag period. Likewise, a partial inhibition or delay in onset of pinocytosis in tissue that had been exposed to leupeptin, (which would cause a delayed delivery of $^{125}I\text{-}BSA_{fd}$ to the lysosomes, so delay degradation and give rise to the lag phase), can be dismissed because pre-incubation of tissue with leupeptin did not affect the uptake of the pinocytic marker $^{125}I\text{-}PVP$. Lysosomal turnover during the lag phase was considered unlikely during the period involved, as discussed above.

The observed lag followed by an increased rate of degradation could be explained by displacement of leupeptin from its site of action, (ie the active site of cysteine cathepsins) by incoming $^{125}I\text{-}BSA_{fd}$, once the $^{125}I\text{-}BSA_{fd}$ reached a sufficiently high concentration within lysosomes. The ability of a substrate to displace an inhibitor is characteristic of competitive inhibitors and

would depend on the dissociation constants of $^{125}$I-BSA$_{fd}$ and leupeptin for lysosomal enzymes. However, since the concentrations of $^{125}$I-BSA$_{fd}$ and leupeptin within the lysosomes are not known, the likelihood of displacement of leupeptin from the lysosomal enzymes cannot be predicted.

An alternative explanation, which is well supported by evidence from the literature, is that a delay in fusion between lysosomes and pinosomes was induced by leupeptin. This would cause a delay in the delivery of $^{125}$I-BSA$_{fd}$ to lysosomes, and hence a delay in onset of degradation would occur. This would not be detectable when $^{125}$I-PVP uptake was studied since accumulation of this marker could occur in either pinosomes or secondary lysosomes. Delayed fusion between endosomes and lysosomes has been proposed as the cause of the observed accumulation of autophagosomes in leupeptin-treated cells. Leupeptin causes an accumulation of undegraded proteins within the lysosomes. Fusion between the distended lysosomes/autophagolysosomes that are caused by this massive protein accumulation and other vesicles is then inhibited, hence giving rise to the observed accumulation of autophagosomes (Korvacs *et al.*, 1982; Furono *et al.*, 1982 a,b). The process of accumulation of proteins and inhibition of fusion is reported to take at least 30 minutes after onset of inhibition of degradation (Tolleshaug & Berg, 1981).

Inhibition of fusion can explain many of the characteristics of the lag period. The amount of material that accumulated in lysosomes would depend on the amount of leupeptin present, and on the period of accumulation. This is dictated by the pre-incubation regime and by the period of 'recovery' allowed. Hence the lag period would be expected to depend on the pre-incubation regime, and would not be prevented by a recovery period.

The studies with inhibitors of pinocytosis were carried out to

assess what proportion of leupeptin was taken up by pinocytosis. Many inhibitors cited in the literature as inhibiting pinocytosis were unsuitable for use, since they were either insufficiently potent or their effects were not reversible. It was important that any inhibitor used should have both these properties. (An incomplete inhibition of pinocytosis would lead to an underestimate of the amount of leupeptin entering the tissue via this route, and incomplete recovery of pinocytosis would lead to an over-estimation of the inhibition of degradation of $^{125}I\text{-}BSA_{fd}$ by leupeptin.) Although many data are available on the effectiveness of pinocytic inhibitors little has been reported on the reversibility of their effects. The inhibitors tested in this study are described below.

Sodium fluoride is reported to inhibit pinocytosis in macrophages by 70% at a concentration of 1mM (Cohn, 1966) by acting as an inhibitor of glycolysis. However, it did not affect pinocytosis in the rat yolk sac at the concentration tested, (20µg/ml). EGTA (1mM) was also without effect. It has been shown to inhibit pinocytosis in the yolk sac by sequestration of $Ca^{2+}$ ions, its effect being reversed when an equal concentration of $CaCl_2$ was included during incubation (Duncan & Lloyd, 1978). Possibly the lack of inhibition observed was due to an excess of $Ca^{2+}$ in medium 199 ($Ca^{2+}$ concentration reported to be 1.8mM.) At 5mM, inhibition of pinocytosis occurred, but recovery was poor. The reason for total lack of recovery in the presence of excess $Ca^{2+}$ (added to replace any sequestered by EGTA) is unknown. Rotenone acts as an inhibitor of the electron transport chain and therefore effects the energy balance of the cell. It has been used in the past to inhibit pinocytosis in rat yolk sac tissue. However, it only induced a 70% inhibition of pinocytosis, and only 50% of this inhibition was reversible. Methylamine (20mM), reported to inhibit pinocytosis by about 90%

(Livesey et al., 1980), blocked the release of TCA soluble radioactivity. However, high levels of radioactivity within the tissue were observed, suggesting that proteolysis rather than pinocytosis was inhibited (as might be expected with this lysosomotropic agent.) Its effects were only partially reversible, even after a prolonged recovery period. (Lack of reversibility of this inhibitor was also observed by Seglen et al., 1979.) Monensin, which acts as a $Na^+$:$K^+$ ionophore (Uchida et al., 1980) is reported to affect endocytosis, however there is some conflict in the literature about the inhibited step. In rat embryo fibroblasts, fluid-phase pinocytosis was inhibited by 90% after 24 hour exposure to monensin (1μM), but fusion between lysosomes and pinosomes was unaffected (Wilcox et al., 1982). Conversly, Harford et al. (1983) found that uptake of an asialoglycoprotein was not inhibited, but subsequent fusion between pinosomes and lysosomes was inhibited by monensin (10μM) in rat hepatocytes. In yolk sacs, monensin (50mM) was shown to decrease the E.I. of $^{125}I\text{-}BSA_{fd}$ by approximately 75%, however the final concentration of 0.1% ethanol in the medium, necessary to solubilize the monensin, was found to adversly affect control tissue. (Such effects have recently been characterised in more detail by G.Steventon; unpublished data). High potassium: low sodium ratios were reported to inhibit endosome-lysosome fusion but not pinocytosis (Baeniziger & Fiete, 1982) However, it appeared to be a very effective inhibitor of uptake of $^{125}I\text{-}BSA_{fd}$ in this system, decreasing the Endocytic Index by approximately 95%. Unfortunately, no study was made on the reversibility of this inhibitor.

Low temperatures have been shown to inhibit pinocytosis in the yolk sac (Duncan & Lloyd, 1978) and in other cells eg perfused liver (Dunn et al., 1980), macrophages (Cohn 1966). In the perfused liver, temperatures below 10°C inhibited endocytosis; between 10 to 20°C

endocytosis occurred slowly, but fusion between pinosomes and lysosomes was prevented. Between 20 to 37°C both pinocytosis and degradation occurred at increasing rates. In yolk sac, pinocytosis was found to be inhibited completely below 20°C. Temperatures of 6, 15, and 20°C were tested for reversibility of their effects and complete inhibition of pinocytosis at 6°C was verified. Proteolytic activity was rapidly regained in all cold-treated tissue. Surprisingly, degradation of $^{125}$I-BSA$_{fd}$ appeared to be stimulated by pre-treatment at a low tempterature. Since uptake of $^{125}$I-BSA$_{fd}$ is the rate-limiting step in its degradation, this suggests that the rate of pinocytosis may have ben stimulated by the cold treatment. Ammonium chloride at a concentration of 20mM was found to fully inhibit proteolysis and pinocytosis. Inhibition was readily reversible, with uptake and proteolysis of $^{125}$I-BSA$_{fd}$ returning to approximately 90% of the control value within 9 minutes. This rapid recovery after inhibition was also reported by Livesey et al.(1980) and Seglen et al. (1979) in hepatocytes.

The properties of low temperature and $NH_4Cl$ (20mM) fulfilled the necessary requirements for this study, therefore these were employed to inhibit pinocytosis during the pre-incubation phase with leupeptin, their effects being reversed during the wash period before incubation with $^{125}$I-BSA$_{fd}$. However, the results obtained with low temperature were ambiguous since, in many cases, the rate of degradation of $^{125}$I-BSA$_{fd}$ in the tissue incubated with leupeptin at low temperature was stimulated above that of the control tissue. Although no conclusion could be reached on the proportion of uptake of leupeptin occurring via pinocytosis, the mechanism of uptake of leupeptin did appear to be temperature dependent. Results obtained using ammonium chloride were more conclusive. Although in several experiments, the effects of $NH_4Cl$ proved to be not totally

reversible, by expressing the rate of degradation of $^{125}I\text{-}BSA_{fd}$ as a percentage of that in the matched control tissue (incubated with or without $NH_4Cl$ in the absence of leupeptin) it was possible to assess the degree of inhibition given by the leupeptin in tissue incubated with both ammonium chloride and leupeptin or just leupeptin. The mean values of percentage residual degradative capacity for tissue treated with leupeptin alone was 57.7%, this increased to 73.4% for tissue incubated with leupeptin in the presence of $NH_4Cl$. This suggests that less leupeptin was able to enter the tissue to cause inhibition of proteolysis in the presence of the inhibitor of pinocytosis. However the specificity of action of both $NH_4Cl$ and low temperature towards pinocytosis as opposed to other possible membrane transport systems is not known. Low temperature reduces the fluidity of the membrane (which may affect entry of leupeptin by diffusion) and would affect any energy-requiring membrane transport such as active transport via a carrier. The mechanism of action of $NH_4Cl$ in the inhibition of pinocytosis is not known, but it is reported to affect cells in many ways (eg see Livesey *et al.*, 1980; Seglen, 1983; Dean *et al.*, 1984). It could therefore inhibit other membrane transport systems or affect the degree of inhibition given by leupeptin by causing it to be lost more rapidly from the tissue after uptake.

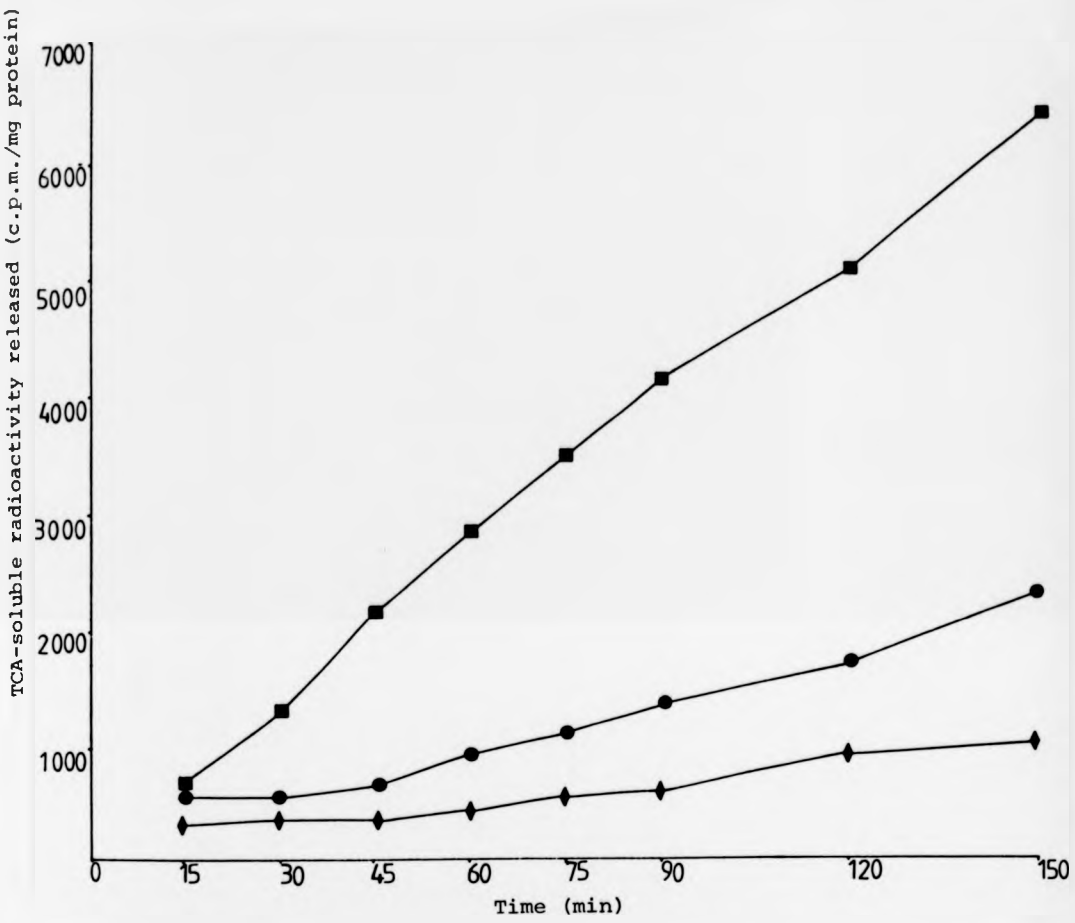



Fig. 3.1 Effect of Leupeptin on the Time-Course of Release of TCA-soluble $^{125}$I-BSA$_{fd}$ Degradation Products

Tissue was pre-incubated with or without leupeptin for 1h then rinsed and transferred to medium containing a trace quantity of $^{125}$I-BSA$_{fd}$, with or without leupeptin as described in Section 3.2.1. The release of TCA-soluble radioactivity was monitored as described in Sections 3.2.1 and 3.2.2. The graph shows typical results.

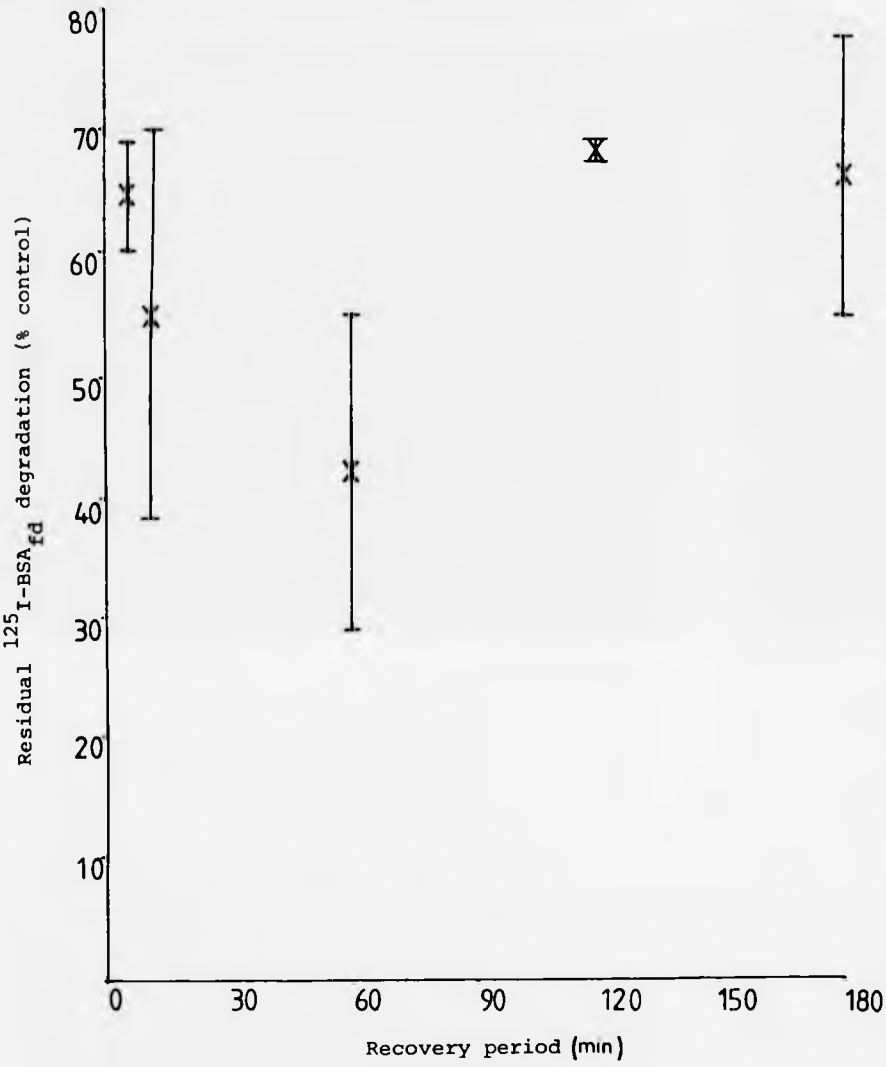



Fig. 3.2 Recovery of Degradative Capacity Towards $^{125}$I-BSA$_{fd}$ Following Exposure to Leupeptin

After incubation with leupeptin (100 µg/ml, 45 min) tissue was rinsed and incubated in substrate-free medium for various periods. Tissue was then transferred to medium containing $^{125}$I-BSA$_{fd}$, and production of TCA-soluble radioactivity monitored as described in Section 3.2.1 . Results are expressed as the mean rate of $^{125}$I-BSA$_{fd}$ degradation ± standard deviation.

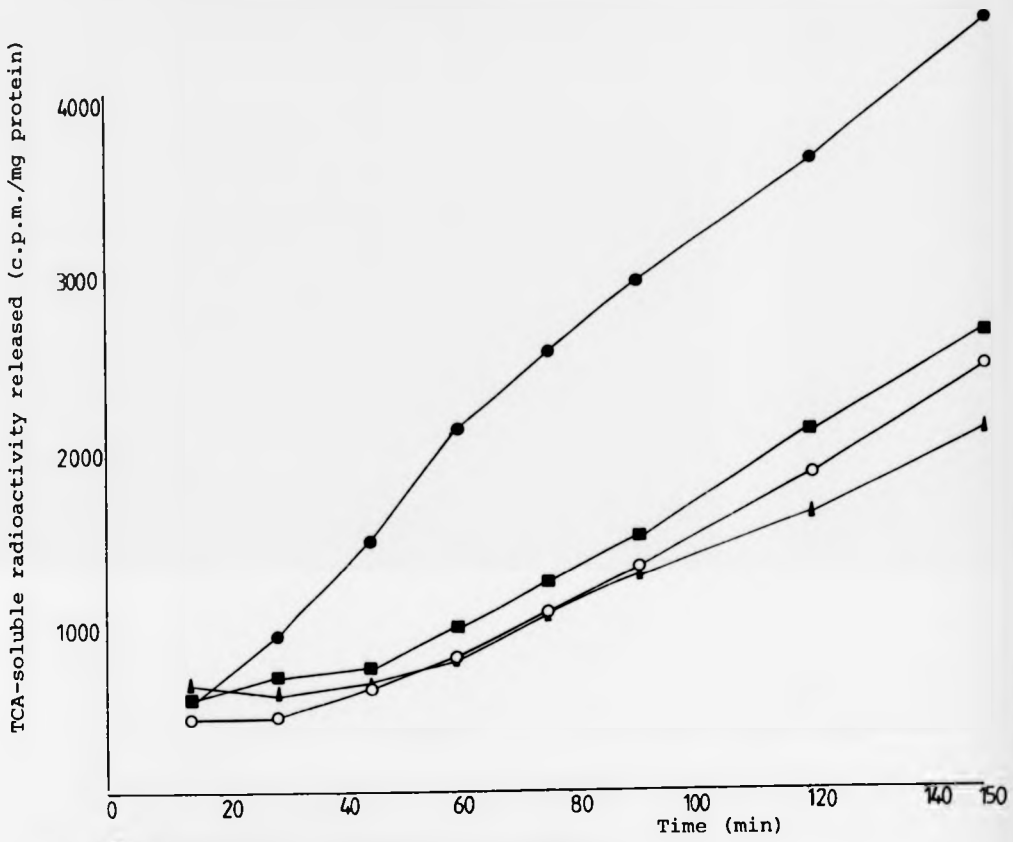



Fig. 3.3 Typical Time-Course of Release of TCA-soluble Radioactivity from Leupeptin-Treated Yolk Sacs Following Various Periods of Recovery

Yolk sacs were treated as described for Fig. 3.2. Results shown are typical time-courses of release of TCA-soluble radioactivity, from which the duration of the lag period (before onset of $^{125}I\text{-}BSA_{fd}$ degradation) and the rate of $^{125}I\text{-}BSA_{fd}$ degradation could be determined.

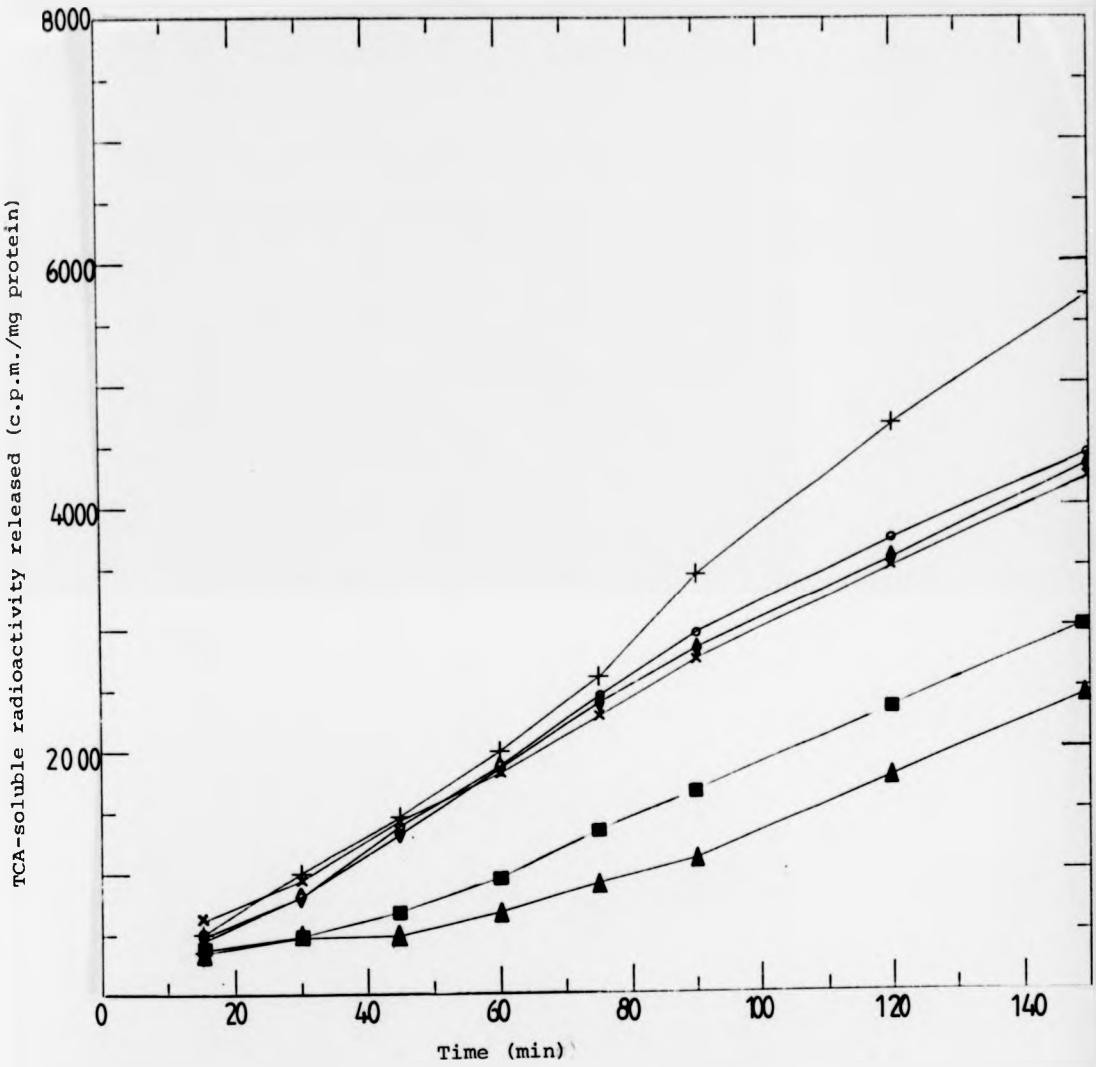



Fig. 3.4 Time-Course of Release of TCA-soluble Radioactivity from Tissue Pre-incubated in Leupeptin at 25 µg/ml for Various Periods

Tissue was pre-incubated with leupeptin (25 µg/ml) for various periods, then rinsed and transferred to leupeptin-free medium containing $^{125}I\text{-}BSA_{fd}$. The release of TCA-soluble radioactivity was monitored as described in Section 3.2.1. Typical results are shown.

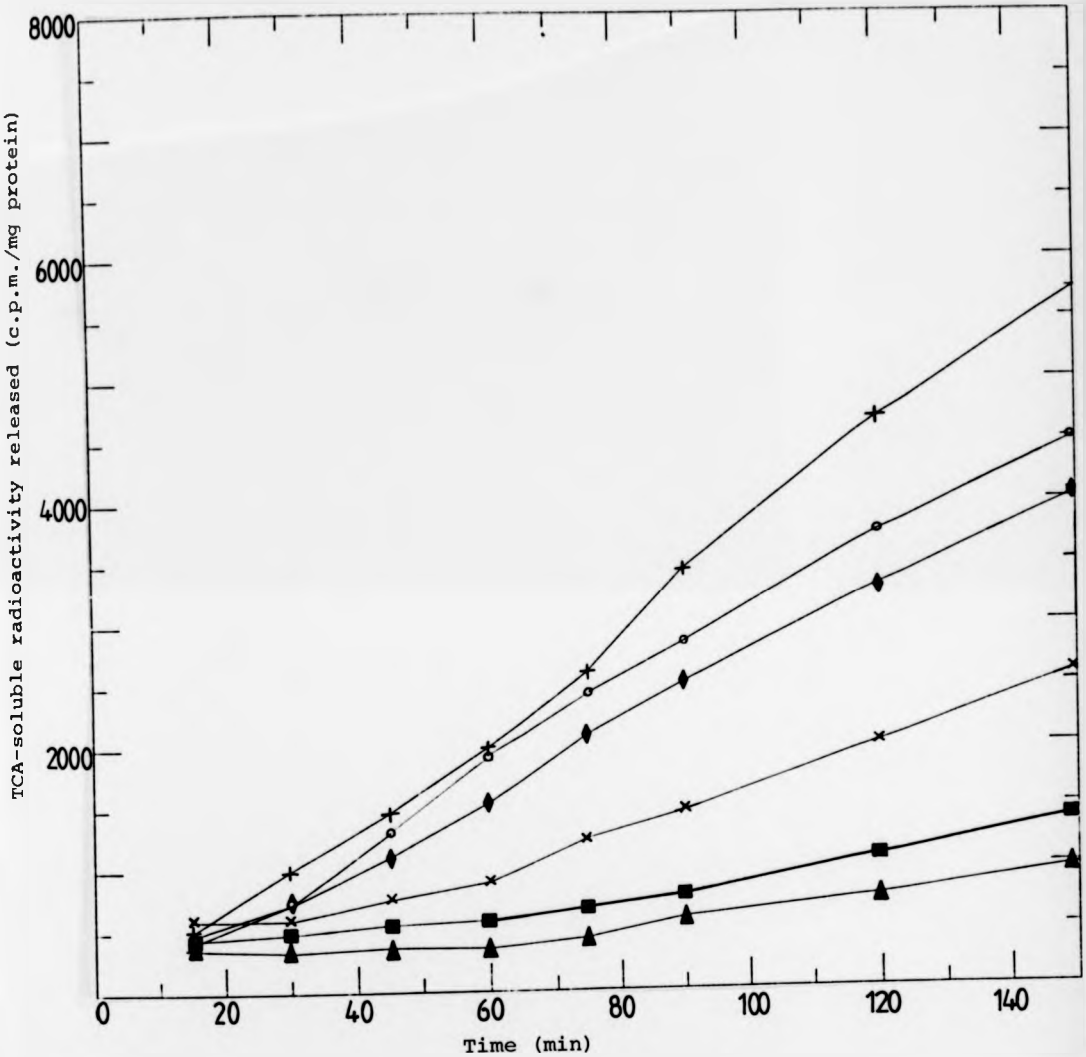



Fig. 3.5 Time Course of Release of TCA-soluble Radioactivity from Tissue Pre-Incubated in Leupeptin at 50 µg/ml for Various Periods

Details as for Fig. 3.4, except that leupeptin was present at a concentration of 50 µg/ml during pre-incubation.

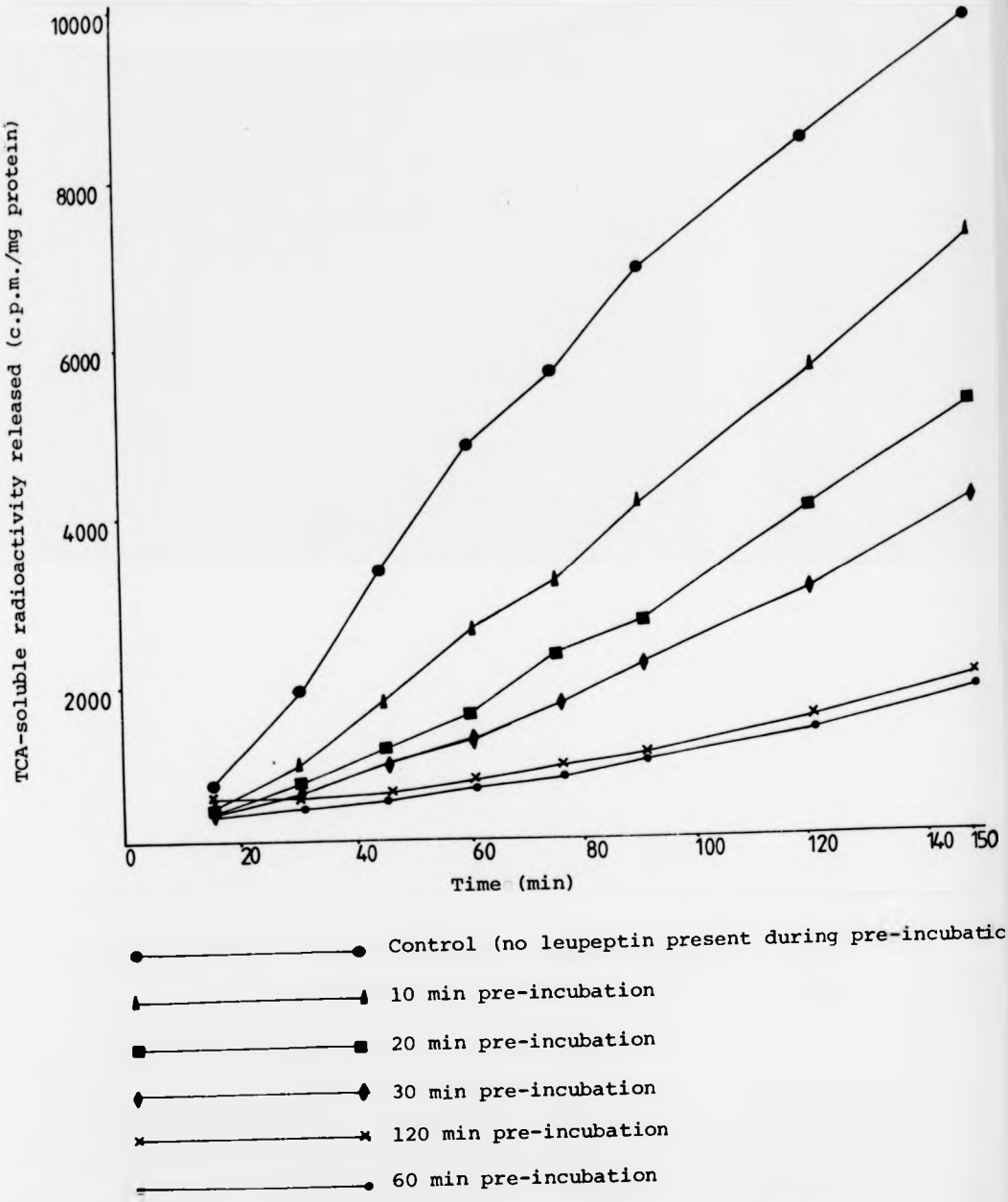



Fig. 3.6 Time-Course of Release of TCA-soluble Radioactivity from Tissue Pre-Incubated in Leupeptin at 100 µg/ml for Various Periods

Details as for Fig. 3.4 except that leupeptin was present at a concentration of 100 µg/ml during pre-incubation

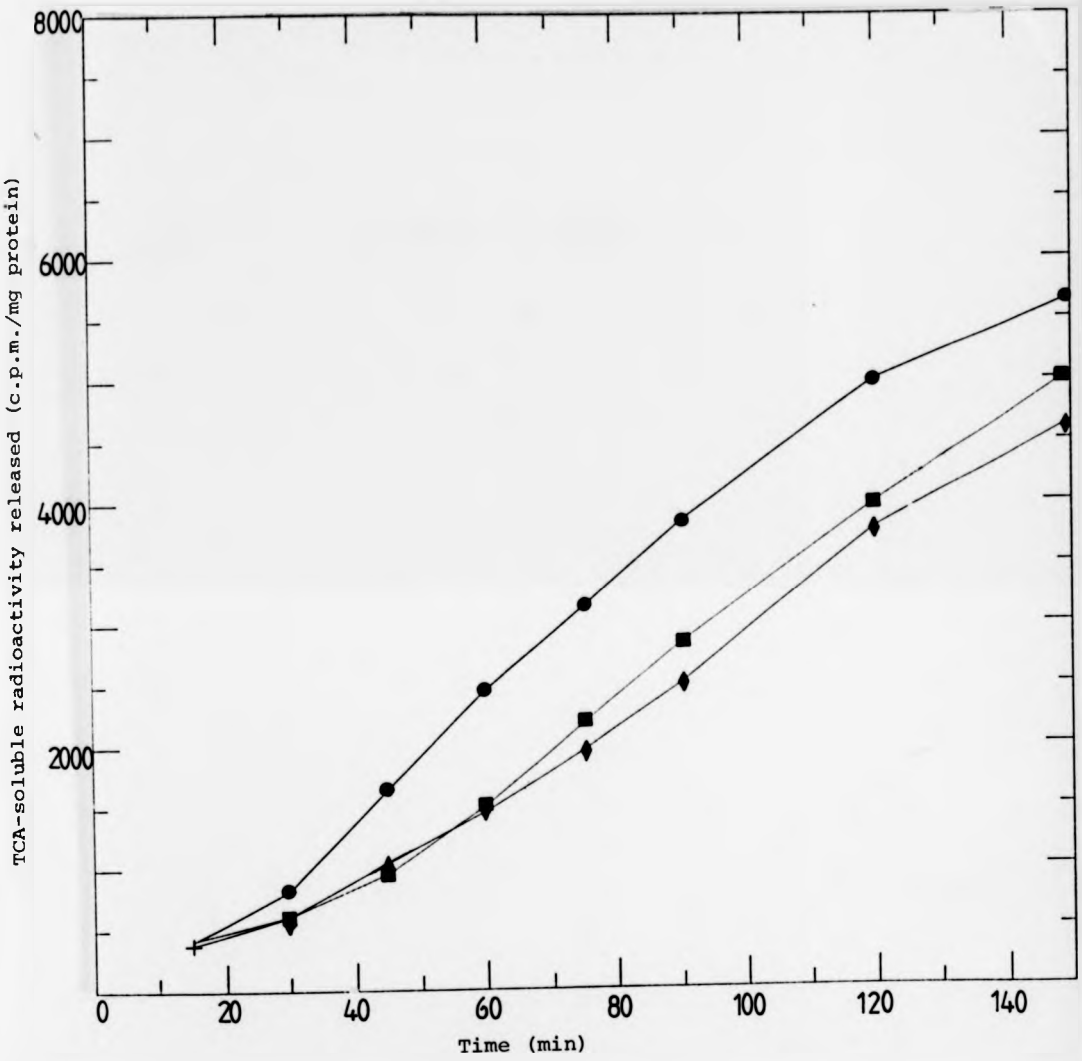
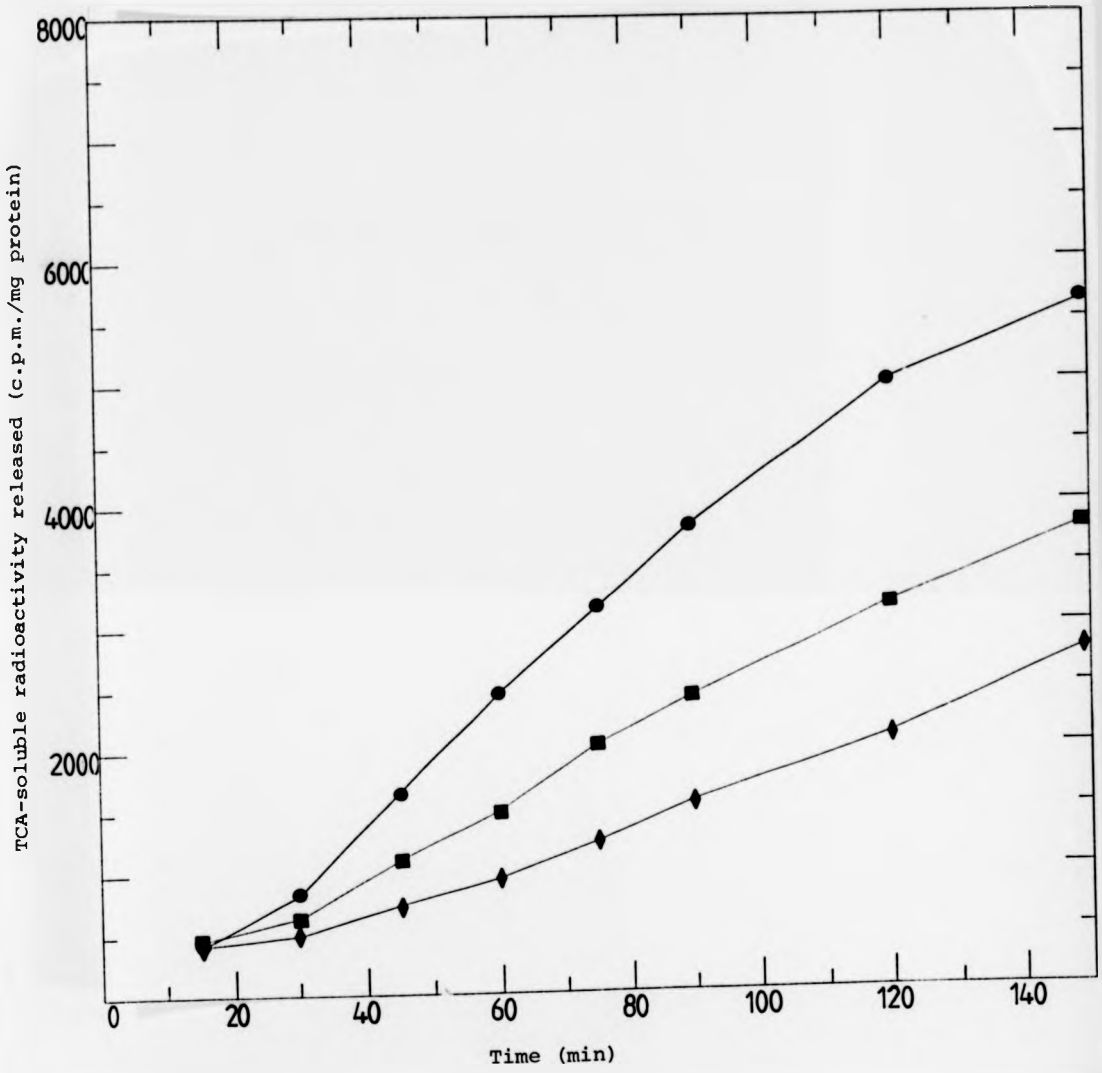



Fig. 3.7a Comparison of the Time-Course of Release of TCA-soluble Radioactivity from Yolk Sacs Pre-Incubated for 10min with Leupeptin at 50 and 100 µg/ml

Tissue was pre-incubated for the given time with leupeptin at 50 and 100 µg/ml, then rinsed and re-incubated in leupeptin-free medium containing $^{125}$I-BSA$_{fd}$. Release of TCA-soluble radioactivity was monitored as described in Section 3.2.1. Typical results are shown.



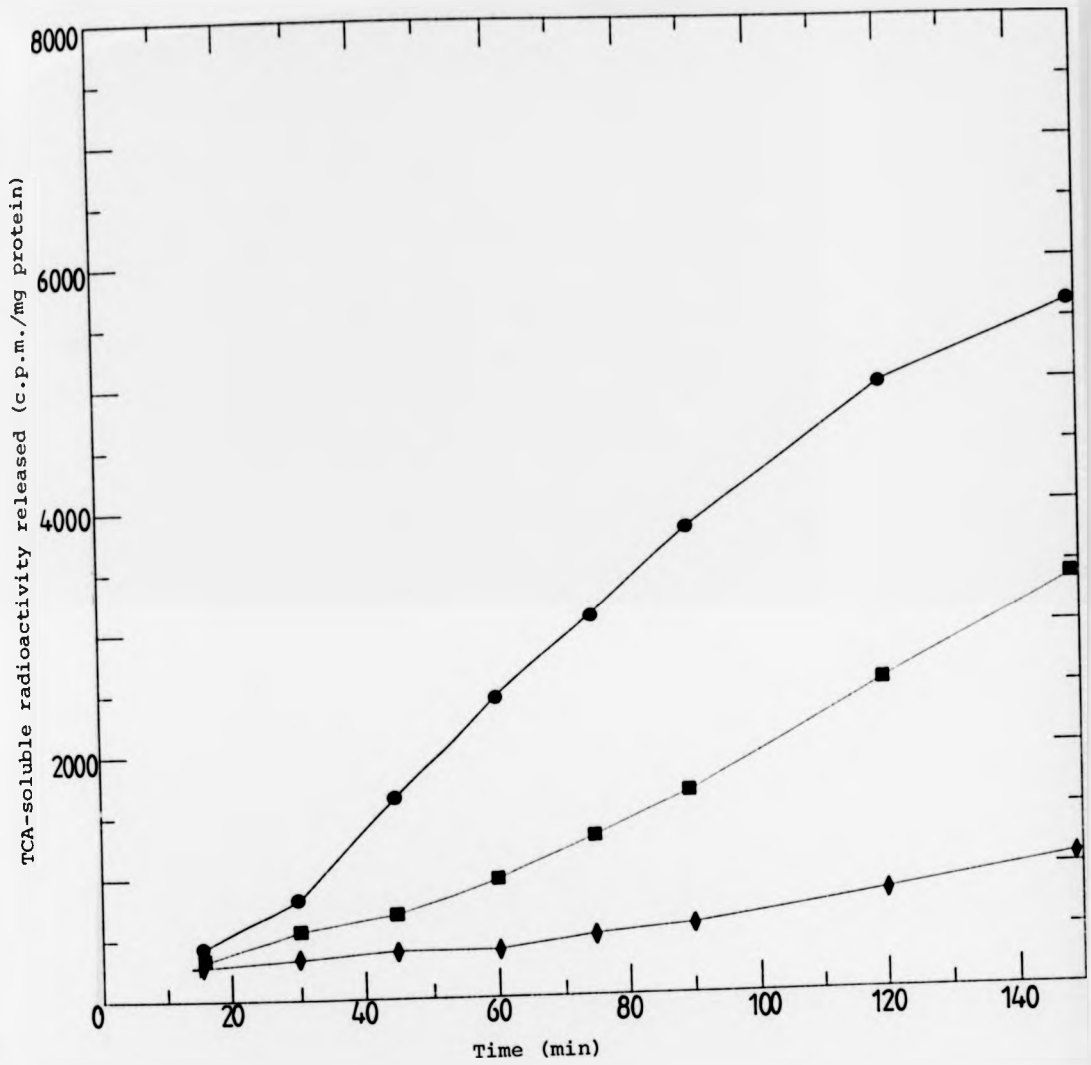

● Control (no leupeptin)

■ Leupeptin (50 µg/ml)

♦ Leupeptin (100 µg/ml)

Fig. 3.7b Comparison of the Time-Course of Release of TCA-soluble Radioactivity from Yolk Sacs Pre-Incubated for 30 min with Leupeptin at 50 and 100 µg/ml

Details as for Fig. 3.7a



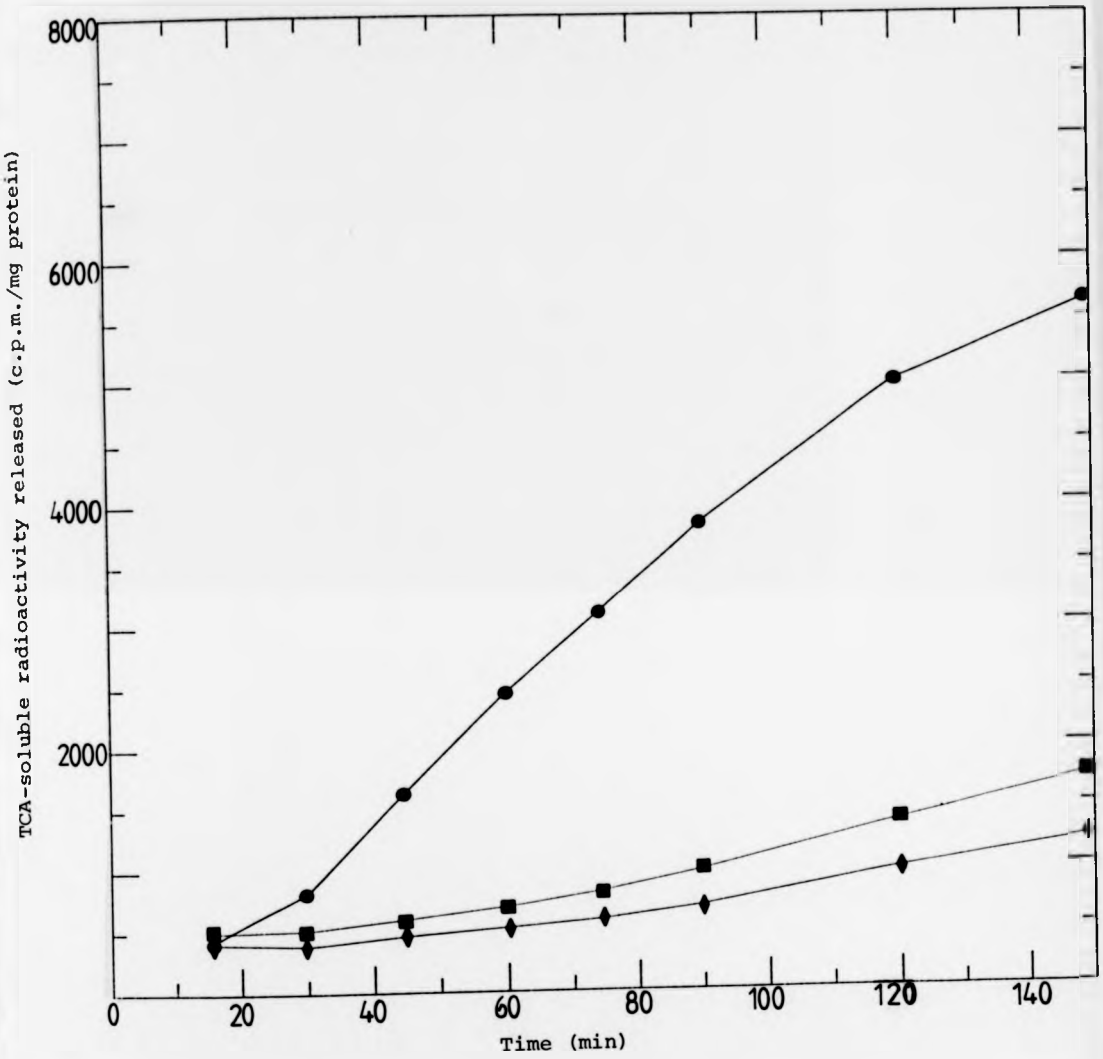

● Control (no leupeptin)

■ Leupeptin (50 µg/ml)

♦ Leupeptin (100 µg/ml)

Fig. 3.7c Comparison of the Time-Course of Release of TCA-soluble Radioactivity from Yolk Sacs Pre-incubated for 60 min with Leupeptin at 50 and 100 µg/ml

Details as for Fig. 3.7a



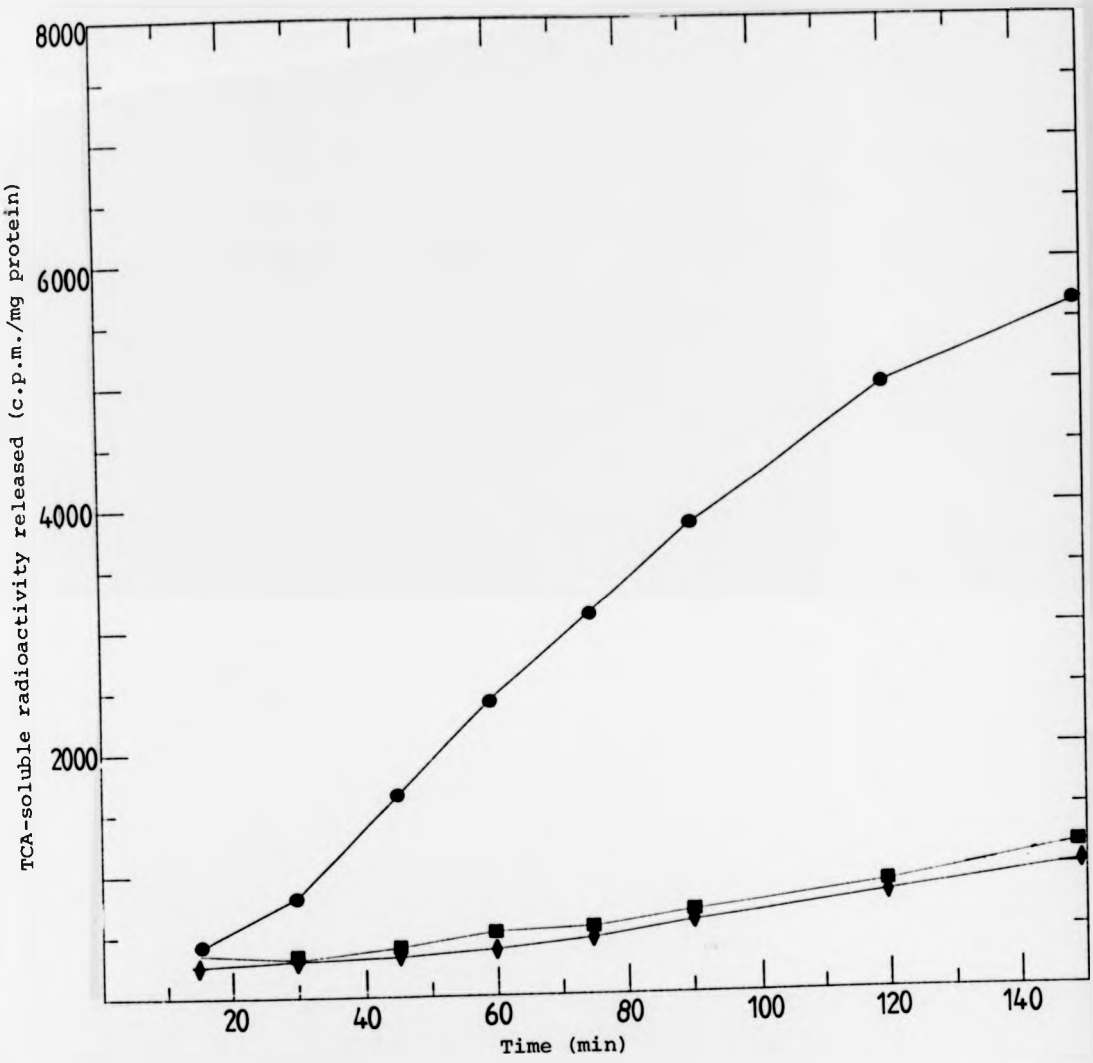

● Control (no leupeptin)

■ Leupeptin (50 µg/ml)

♦ Leupeptin (100 µg/ml)

Fig. 3.7d Comparison of the Time-Course of Release of TCA-soluble Radioactivity from Yolk Sac Pre-Incubated for 120 min with Leupeptin at 50 and 100 µg/ml

Details as for Fig. 3.7a



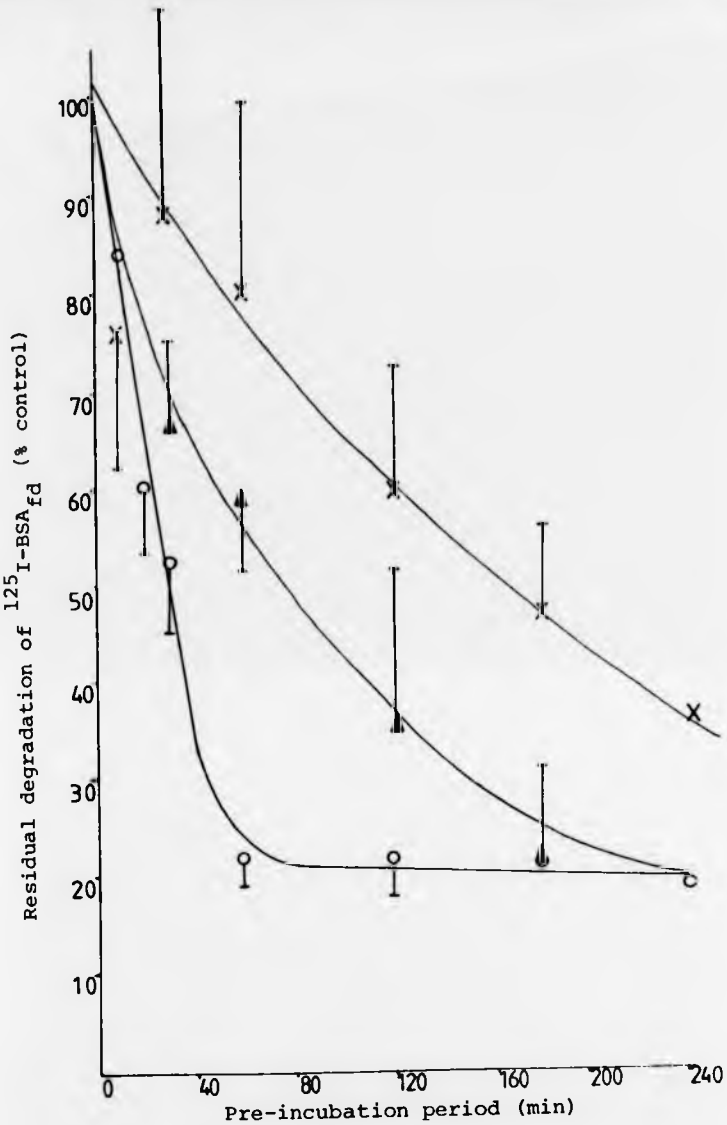

● Control (no leupeptin)

■ Leupeptin (50 µg/ml)

♦ Leupeptin (100 µg/ml)

Fig. 3.7e Comparison of the Time-Course of Release of TCA-soluble Radioactivity from Yolk Sacs Pre-Incubated for 180 min with Leupeptin at 50 and 100 µg/ml

Details as for Fig. 3.7a




X Leupeptin at 25 µg/ml present during pre-incubation

▲ Leupeptin at 50 µg/ml present during pre-incubation

o Leupeptin at 100 µg/ml present during pre-incubation

Fig. 3.8 Effect of Leupeptin Concentration and Period of Exposure to Leupeptin on the Subsequent Rate of Degradation of $^{125}$I-BSA$_{fd}$

The rate of release of TCA-soluble radioactivity from tissue treated with leupeptin as described in Fig. 3.4, 3.5 and 3.6 was determined, and expressed as a percentage of that of control tissue that had not been exposed to leupeptin. The mean (± standard deviation) percentage inhibition of release is given for each concentration of leupeptin for various pre-incubation periods.

Fig. 3.9a

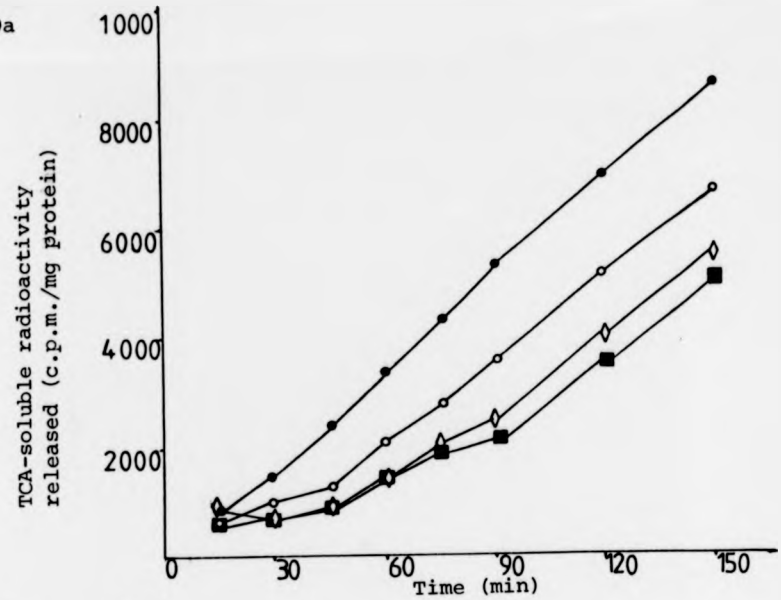
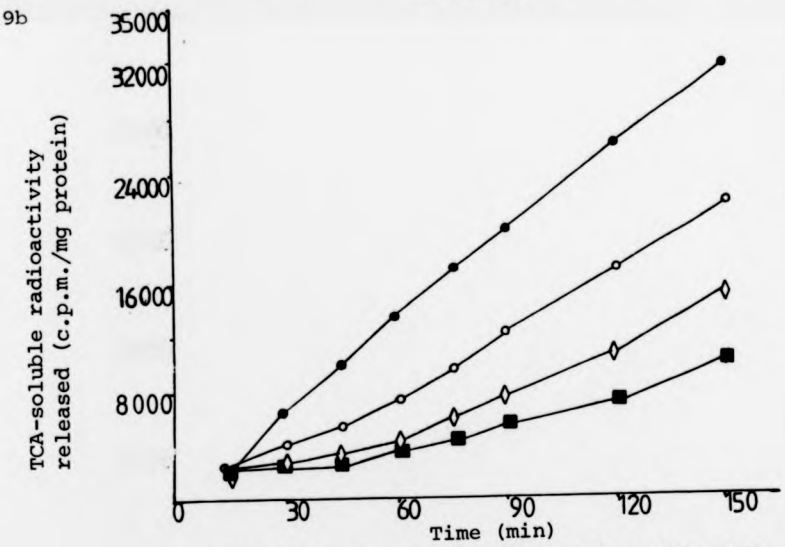



Fig. 3.9b




- Control (no leupeptin or ammonium chloride)
- Ammonium chloride (20mM) present during pre-incubation
- Ammonium chloride (20mM) and Leupeptin (100 µg/ml) present
- Leupeptin (100 µg/ml) present during pre-incubation

Fig. 3.9a,b Time-Course of Release of TCA-soluble Radioactivity from Tissue Pre-Incubated with Leupeptin in the Presence and Absence of Ammonium Chloride

Yolk sacs were pre-incubated for 45min with leupeptin in the presence and absence of ammonium chloride. The tissues were then rinsed and re-incubated with $^{125}I\text{-}BSA_{fd}$, and the release of TCA-soluble material monitored as described in Section 3.2.1. Each diagram represents results from a single experiment.

Fig. 3.9c

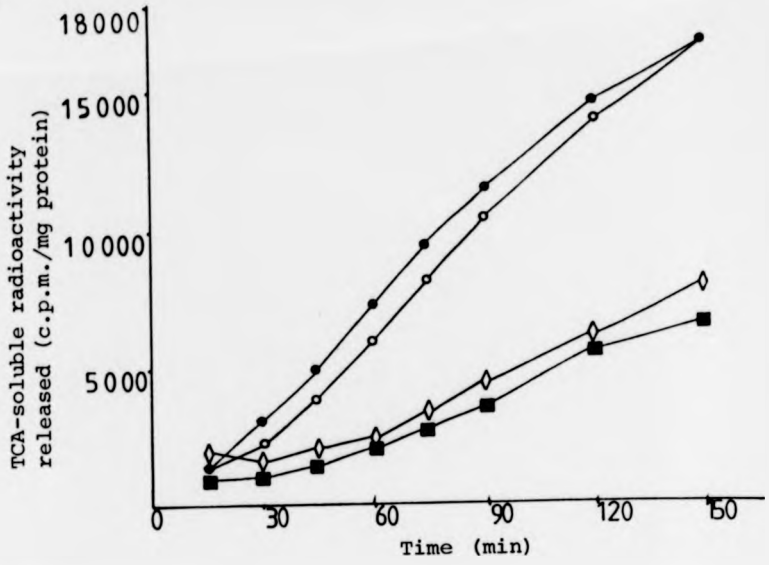
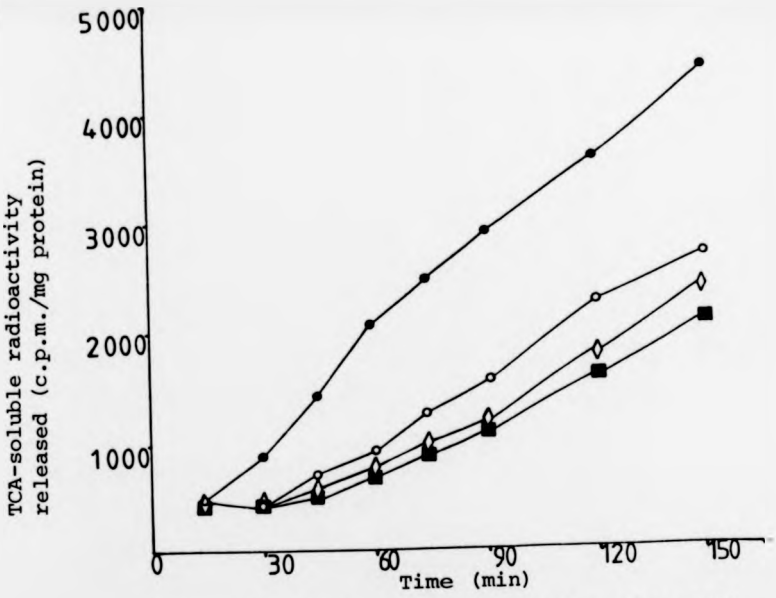



Fig. 3.9d




- Control (no leupeptin or ammonium chloride)
- Ammonium chloride (20mM) present during pre-incubation
- Ammonium chloride (20mM) and Leupeptin (100 µg/ml) presen
- Leupeptin (100 µg/ml) present during pre-incubation

Fig. 3.9c,d Time-Course of Release of TCA-soluble Radioactivity from Tissue after Pre-Incubation with Leupeptin in the Presence and Absence of Ammonium Chloride

Details as for Fig. 3.9a,b

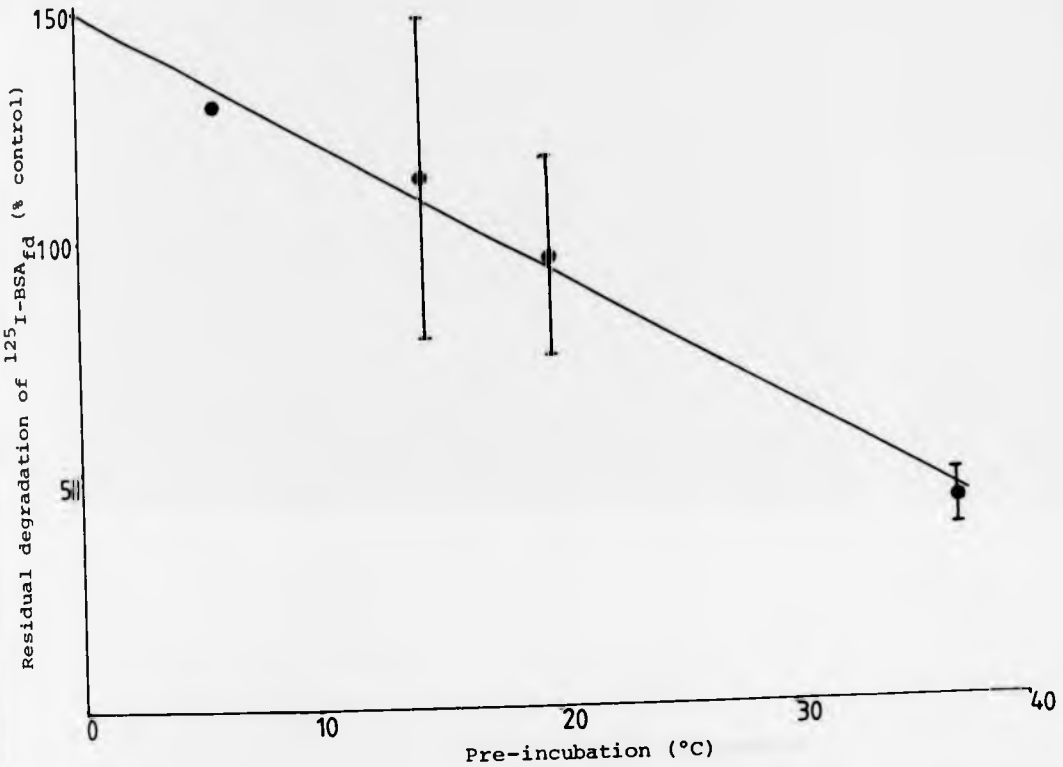



Fig. 3.10 Effect of Temperature of Pre-Incubation with Leupeptin (100μg/ml) on the Subsequent Rate of Degradation of $^{125}$I-BSA$_{fd}$

Yolk sacs were pre-incubated with leupeptin for 60 min at the temperatures indicated. A control, in which no leupeptin was present during preincubation, was maintained at 37°C. The tissues were rinsed and reincubated with $^{125}$I-BSA$_{fd}$ at 37°C. The rate of release of TCA-soluble radioactivity from leupeptin-treated tissue was determined, and calculated as a percentage of that from control tissue. The points represent the mean values ± standard deviation at each temperature.

Table 3.1

Degradation of $^{125}I\text{-}BSA_{fd}$ after 1h Pre-incubation With or Without Leupeptin (100 µg/ml)

Tissue was incubated as described in Section 3.2.1. The rate of $^{125}I\text{-}BSA_{fd}$ degradation was determined from the slope of the linear portion of the time-course of release of TCA-soluble radioactivity.

| Experiment | Rate of $^{125}I\text{-}BSA_{fd}$ Degradation (cpm/min/mg yolk-sac protein) | | Residual Degradative Capacity |
|---|---|---|---|
| | Pre-incubated with leupeptin (100 µg/ml) | Pre-incubated without leupeptin (control) | |
| i | 25.8 | 56.7 | 45.5 |
| ii | 12.4 | 53.2 | 23.3 |
| iii | 16.2 | 67.5 | 24.0 |
| iv | 14.2 | 77.2 | 18.4 |
| v | 21.1 | 47.7 | 44.2 |
| vi | 40.9 | 86.7 | 47.2 |
| vii | 19.2 | 58.0 | 33.1 |
| viii | 13.2 | 39.9 | 33.1 |
| ix | 19.2 | 58.0 | 33.1 |

Table 3.2

Effectiveness of Inhibitors of Pinocytosis in the Rat Yolk Sac

Table 3.2a

The effect of inhibitors on the rate of uptake and degradation of $^{125}I\text{-}BSA_{fd}$ was monitored by measuring the rate of release of TCA-soluble $^{125}I\text{-}BSA_{fd}$ degradation products, as described in Section 3.2.6a. Results are from typical experiments.

| Inhibitor | Concentration | Rate of $^{125}I\text{-}BSA_{fd}$ Degradation (cpm/min/mg yolk-sac protein) | | Remaining Pinocytic/ Degradative Capacity (%) |
|---|---|---|---|---|
| | | Inhibitor present | Control | |
| Ammonium Chloride | 20mM | 0.0 | 21.0 | 0.0 |
| Low Temperature | 6°C | 0.87 | 86.7 | 1.0 |
| Methylamine | 20mM | 1.4 | 21.4 | 6.5 |
| Rotenone | $10^{-5}$M | 23.2 | 86.7 | 26.8 |
| Ammonium Chloride | 10mM | 14.9 | 32.8 | 45.5 |
| EGTA | 1mM | 40.1 | 39.9 | 100.5 |
| Sodium Fluoride | 20 µg/ml | 24.1 | 21.4 | 112.6 |

Table 3.2b

The effect of inhibitors on the Endocytic Index of $^{125}I\text{-}BSA_{fd}$ was determined as described in Section 3.2.6a.

| Inhibitor | Concentration | EI of $^{125}I\text{-}BSA_{fd}$ | | Remaining Pinocytic Activity (%) |
|---|---|---|---|---|
| | | Inhibitor present | Control | |
| Monensin | 50mM | 67.7 | 262.4 | 25.8 |
| High $K^+$:$Na^+$ | | 10.9 | 288.3 | 3.8 |

Table 3.3

Reversibility of Inhibitors of Pinocytosis in the Rat Yolk Sac

Tissue was incubated in the presence of inhibitors of pinocytosis for 1h, then rinsed and/or allowed to recover in the absence of inhibitor, and re-incubated in medium containing $^{125}I\text{-}BSA_{fd}$, as described in Section 3.2.6b. Typical rates of release of TCA-soluble radioactivity from inhibitor-treated and control tissue are given together with the percentage recovery.

| Inhibitor | Conc. | Rinse/Recovery Period (min) | Rate of Release of TCA-Soluble Radioactivity (cpm/min/mg protein) | | Recovery (%) |
|---|---|---|---|---|---|
| | | | Inhibitor-treated | Control | |
| Ammonium Chloride | 20mM | 9 | 53.9 | 56.4 | 95.6 |
| | | 12 | 28.5 | 35.9 | 73.0 |
| | | 30 | 14.6 | 14.9 | 98.0 |
| Low Temperature | 6°C | 4 | 121.0 | 86.7 | 140.5 |
| | 15°C | 4 | 41.5 | 44.2 | 93.9 |
| | 20°C | 4 | 36.4 | 44.2 | 82.4 |
| Methylamine | 20mM | 12 | 0.0 | 26.9 | 0.0 |
| Rotenone | $10^{-5}$M | 30 | 44.3 | 86.7 | 51.1 |
| EGTA* | 5mM | 12 | 0.0 | 44.2 | 0.0 |
| EGTA | 5mM | 12 | 28.3 | 80.7 | 35.1 |

*Calcium chloride (5mM) present during re-incubation period.

Table 3.4

Effect of Ammonium Chloride on the Leupeptin-Induced Decrease in the Rate of $^{125}I\text{-}BSA_{fd}$ Degradation

The rate of release of TCA-soluble radioactivity after pre-incubation with leupeptin in the presence of ammonium chloride was expressed as a percentage of that measured after preincubation with ammonium chloride alone, and the rate of release of TCA soluble radioactivity after pre-incubation with leupeptin alone expressed as a percentage of that measured after pre-incubation in inhibitor-free medium.

| Experiment | Rate (cpm/min/mg protein) after pre-incubation with: | | Residual Degradative Capacity | Rate (cpm/min/mg protein) after pre-incubation with: | | Residual Degradative Capacity |
|---|---|---|---|---|---|---|
| | Ammonium chloride plus Leupeptin | Ammonium chloride | | Leupeptin | No inhibitor | |
| a | 48.5 | 53.9 | 90.0 | 42.1 | 56.4 | 74.6 |
| b | 114.5 | 157.9 | 72.5 | 74.8 | 203.2 | 36.8 |
| c | 60.9 | 123.6 | 49.3 | 49.9 | 107.0 | 46.6 |
| d | 16.7 | 20.6 | 81.1 | 19.0 | 26.0 | 73.1 |

# CHAPTER 4

# CHROMOGENIC METHOD OF LEUPEPTIN DETECTION

## 4.1. INTRODUCTION

The rate of uptake of a substrate can give a good indication of the mode of entry of the substrate into cells. If the uptake kinetics of the test substrate are similar to those of a standard marker substrate, whose uptake kinetics and mode of uptake are established in the same cell type, uptake of the test substrate is probably, but not definitely, by the same mechanism (Roberts *et al.*, 1977; Shaw & Dean, 1980). In some cases, competition between substrates and saturability characteristics can also be used to identify the mode of uptake, eg amino acid active transport systems (reviewed by Christensen, 1984).

In the previous chapter inhibition of intra-lysosomal proteolysis was used as the basis of a semi-quantitative method of estimating the rate of entry of leupeptin into the lysosomal system of yolk-sac cells. However, a number of assumptions and problems are encountered in trying to derive a rate of uptake of leupeptin from such data (see Chapter 3 for further discussion).

An established method of comparing the rates of uptake of different substrates that are captured by pinocytosis is to compare values of the Endocytic Index (E.I.), which is equivalent to the volume of external fluid (µl) whose substrate content has been internalized by unit quantity of tissue (mg protein) per unit time (h); Williams *et al.* (1975a). Rates of uptake expressed in this way may be compared regardless of differences in day-to-day specific radioactivity or method of detection of the substrate (eg Roberts *et al.*, 1977, compared $^{125}I$-, $^{14}C$- and $^{198}Au$-labelled substrates).

The basal rate of uptake is given by the rate of capture of a marker that enters entirely by fluid phase pinocytosis. If the E.I. of a substrate is greater than that of markers for fluid-phase pinocytosis, the substrate may have an affinity for the plasma

membrane (via binding to specific receptors or to non-specific adsorption sites) so that it is taken up both in the fluid-phase and bound to the internalized membrane (ie. adsorptive pinocytosis). An alternative possibility, available for small or lipophilic molecules, is that the substrate enters by passive diffusion or facilitated or active transport. The net rate of uptake would then be a composite of the rate of pinocytosis and the rates of accumulation via other membrane transport systems. [A rate of uptake lower than that of fluid phase pinocytosis would not be expected because in yolk-sac tissue pinocytosis occurs continuously, thus substrate present in the extracellular fluid must enter the cell at the rate of fluid-phase pinocytosis (unless the agent modifies the rate of pinocytosis at tracer concentrations).]

Comparison of the uptake of leupeptin with that of markers of fluid pinocytosis would thus indicate whether uptake occurred at the same rate as fluid-phase pinocytosis or was accelerated as a result of either adsorption to the plasma membrane or uptake via additional membrane transport systems. Several such markers for fluid-phase and adsorptive pinocytosis are known. These are all molecules that enter the cell by pinocytosis alone because they are either too large and/or too polar to enter by membrane permeation. In rat yolk sacs, $^{125}$I-PVP has been used extensively; $^{14}$C-sucrose and certain preparations of colloidal ($^{198}$Au) gold are also taken up in the fluid phase (Roberts et al., 1977).

Values of the rate of uptake obtained by measuring the substrate content of the tissue will not automatically give a true indication of the rate of entry of the substrate. Non-degradable macromolecular substrates entering the tissue by pinocytosis will remain trapped within lysosomes, therefore the rate of accumulation in the tissue of such a substrate is equivalent to the rates of capture by

pinocytosis (provided there us no appreciable loss via exocytosis). However, when degradable substrates enter lysosomes, they yield fragments that are able to pass out of the tissue (eg amino acids, mono-saccharides). The amount of substrate remaining within the tissue at any time will be less than the total amount that has been captured, as a result of loss by degradation. If the rates of uptake and degradation are equal (as would be the case when entry is the rate-limiting step) the net rate of accummulation will fall to zero and a steady-state concentration of substrate within the tissue will be observed (Williams et al., 1975b; Livesey & Williams, 1979). A steady-state concentration could also be observed if the intact substrate was rapidly lost from the tissue by diffusion or membrane transport. Hence a linear time-course of accumulation of leupeptin would only be expected if inactivation, degradation or loss from the tissue occurred at rates far lower than the rate of uptake. If a steady-state concentration was observed, uptake followed by inactivation and/or loss by membrane permeation, may be responsible.

In this and subsequent chapters, attempts were made to determine the rate of accumulation of leupeptin in order to gain insight into its mode of uptake and intracellular fate.

The method used to detect leupeptin throughout this chapter is based on the original assay technique developed by Umezawa and co-workers (eg Aoyagi & Umezawa, 1975; Umezawa & Aoyagi, 1977), and has been successfully modified by others (eg Beynon et al., 1981). It relies on the ability of leupeptin to inhibit the proteinase trypsin. To estimate leupeptin concentrations, the rate of hydrolysis of the trypsin substrate benzoyl-arginine-p-nitroanilide (BAPNA) was determined in the presence and absence of a sample of leupeptin. The percentage inhibition observed could be used to estimate the

amount of leupeptin present by comparison with a standard curve (see Beynon et al., 1981). The leupeptin content of tissue that had been incubated with leupeptin, and the medium in which the tissue was incubated, can both be analysed by this method; hence uptake could be calculated in terms of µl/mg protein. The assay detected the content of active leupeptin in the whole tissue, (not just that within secondary lysosomes, c.f. Chapter 3). Inactivated or fully degraded leupeptin would not be detected by this method.

The method of assay was also used to follow the fate of leupeptin in yolk-sac tissue. There are many reports in the literature that suggest leupeptin may be inactivated within tissue (see Section 4.4.5. for further comment), therefore leupeptin was incubated with a yolk-sac homogenate in an attempt to characterise its inactivation.

Rapid inactivation of leupeptin (in the incubation medium) by proteolytic enzymes released from the yolk sac could cause a rapid depletion in the concentration of leupeptin to which the yolk-sacs were exposed. The stability of leupeptin in "conditioned" medium (ie medium in which tissue had been incubated) was therefore also investigated.

In summary the main aims of the experiments reported in this chapter were to investigate:-

i) The rate of accumulation of active leupeptin by yolk-sac tissue.

ii) The rate and pH optimum of degradation of leupeptin by yolk-sac homogenates.

iii) The stability of leupeptin in "conditioned" medium.

## 4.2. MATERIALS AND METHODS

### I. MATERIALS

**Equipment**

Spectrophotometer — 550 S UV/VIS Spectrophotometer.
Perkin Elmer, Beaconsfield, Bucks U.K.
Sample chamber thermostatted to 25°C.

Chart Recorder — Hitachi Ltd. Model 056-1002.
Tokyo, Japan.

All other equipment as described in Chapter 2.

**Reagents**

BAPNA — N-Benzoyl-DL-Arginyl-4-Nitroanilide,
Sigma Chemical Co., Poole, Dorset, U.K.
1mg/ml solution, dissolved in hot (approximatly 90°C) distilled water.

Triethanolamine buffer: — Triethanolamine hydrochloride (0.2M), adjusted to pH 7.8 with sodium hydroxide, containing calcium chloride (20mM).

Trypsin: — 50µg/ml solution, dissolved in HCl acidified water, pH 3.

All other reagents as described in Chapter 2.

## II. METHODS

### 4.2.1. Leupeptin Assay: Inhibition of Trysin-Mediated Hydrolysis of N-Benzoyl-DL-Arginyl-4-Nitroanilide (BAPNA)

The method used was developed from that of Beynon et al. (1981), which was itself based on a general trypsin inhibitor assay described by Fritz et al. (1974). The rate of hydrolysis of BAPNA was measured by following the increase in extinction at 405nm caused by the release of p-nitroaniline. By determining the degree of inhibition of this reaction by known amounts of leupeptin, a standard curve relating percentage remaining activity to the amount of leupeptin present in the assay mixture could be constructed, and unknown concentrations determined. Maximal inhibition of trypsin occurred within 3 min therefore it was possible to use the same reaction mixture to measure the rate of hydrolysis both before and after addition of leupeptin. The reaction was performed as follows.

A freshly-prepared solution of BAPNA (0.5ml) was mixed with triethanolamine buffer (1.9ml) in a cuvette, and allowed to equilibrate to 25°C in a water-bath. Trypsin solution (0.1ml) was added to initiate hydrolysis and the increase in extinction at 405nm followed for about one minute using a thermostatted spectrophotometer coupled to a chart recorder. An aqueous solution (10µl) containing leupeptin was then added to the cuvette with mixing, and the new, inhibited rate of reaction monitored, after about 5min, for a further 5min. The slopes of the chart recorder traces were determined to find the rates of the uninhibited and inhibited reactions, and the percentage residual activity in the presence of leupeptin calculated.

A standard curve was prepared by using the data obtained on adding known concentrations of leupeptin to the assay mixture. A plot of percentage residual activity against amount of leupeptin added (µg

per 10µl) was non-linear. A good straight-line transformation of the data was obtained when the probit value of the percentage residual activity was plotted against the log of the amount of leupeptin present. (A discussion on the use of probit plots and probit analysis can be found in Beynon et al., 1981 and in Colquhoun 1971.) The probit value of the percentage residual activity was found from appropriate tables.

### 4.2.2. Accumulation of Leupeptin

The method used to incubate the tissue was similar to that described in Section 2.6., except separate yolk sacs were used to determine the uptake of $^{125}$I-PVP. Hence, two sets of flasks were set up, each flask containing three yolk sacs. One set of flasks contained medium (6.3ml) and leupeptin (0.7ml, 0.5 or 1.0mg/ml) and the other set contained medium (6.3ml) and $^{125}$I-PVP (0.7ml, 20-40µg/ml). In some experiments, the medium contained 10% calf serum. At intervals over 3 to 9h, one flask from each set was removed from the water bath. Tissue and medium from the leupeptin incubation were prepared for assay as described in Section 4.2.4. Tissue and medium from the $^{125}$I-PVP uptake experiment were assayed as described in Section 2.4a. The quantity of tissue-associated leupeptin was calculated as described in Section 2.7d., and the quantity of tissue-associated $^{125}$I-PVP was calculated as described in Section 2.7a.

### 4.2.3. Inactivation of Leupeptin by a Yolk-Sac Homogenate

A yolk-sac homogenate was prepared as follows. Yolk sacs were dissected as described in Section 2.2., then rinsed immediately in ice-cold saline (1%). The tissue was then homogenized in ice-cold distilled water (usually 0.5ml per yolk sac) using a Potter

Elvehjem-type Teflon-on-glass homogenizer. The homogenate was then centrifuged at 1000g for 10 mins to pellet any cellular debris, and the supernatant used for the inactivation investigations.

In experiments in which the effect of pH on leupeptin inactivation was studied the buffers used were:-

| | |
|---|---|
| pH 3.5 and 4.5 | 0.01M Acetic acid/NaOH |
| pH 5.5 and 6.5 | 0.01M Maleic acid/NaOH |
| pH 7.5 | 0.01M $Na_2HPO_4/NaH_2PO_4$ |
| pH 8.5 and 9.5 | 0.01M Glycine/NaOH |

All buffers were made up to an ionic strength of 0.15M with sodium chloride. For these experiments equal volumes (0.5ml) of homogenate-supernatant and buffer were mixed in a 3ml disposable tube, then a sample of leupeptin (50µg) added. The tubes were kept at 37°C for 15-5 then any reaction stopped by the addition of TCA (5% w/v solution, 1ml) to precipitate the homogenate protein. The TCA-soluble fraction (obtained by spinning down the precipitate at 1000g, 20min) was assayed for active leupeptin. In control experiments the stability of leupeptin in the various buffers was examined by using the same method except distilled water replaced the homogenate.

In one experiment to determine the optimum pH of degradation, mercaptoethanol was included in the incubation mixture. Homogenate (0.5ml), buffer (0.4ml) and mercaptoethanol (20mM, 0.1ml) were mixed, then the experiment continued as described above. Control incubations to find the stability of leupeptin over the pH range also included mercapethanol. An additional control, to assess whether the buffers gave sufficient buffering capacity to maintain the desired pH, was also carried out. Because of the low volumes involved, a pH meter could not be used, therefore the pH profile of $^{125}I\text{-}BSA_{fd}$ degradation was monitored. Incubations were carried out as for the leupeptin

incubations, except $^{125}I\text{-}BSA_{fd}$ (0.5ml) was added instead of leupeptin. The percentage of TCA-soluble material generated after incubation was then determined.

In experiments to determine the time-course of leupeptin inactivation, buffer at pH 7.5 was used for all incubations; the reaction was stopped after periods of incubation between 2-30h.

4.2.4. Preparation of Samples for Leupeptin Assay

4.2.4a **Incubated Yolk Sacs** Yolk sacs that had been incubated with leupeptin and rinsed were homogenised in 1.5ml distilled water in a Teflon-on-glass Potter-Elvehjem type homogeniser. The homogenate was then sonicated (30sec) to disrupt any vesicles and centrifuged at 1000g for 20min, to remove cellular and tissue debris. The supernatant was used directly in the leupeptin assay. Samples (2 x 0.1ml) were removed for protein assay. The remaining supernatant and pellet were digested in sodium hydroxide (1M, 5ml) and also assayed for protein content as described in Section 2.3, using 0.05ml digest per assay.

4.2.4b **Incubation Medium** Medium containing leupeptin was diluted with distilled water to give a leupeptin concentration of about 25µg/ml before assay.

4.2.4c **Homogenate** In experiments in which the degradation of leupeptin by yolk-sac homogenate was measured, the incubation was terminated by precipitating the homogenate proteins with an equal volume of aqueous TCA (5% w/v). The TCA-soluble fraction was assayed for leupeptin. The effect of addition of a sample of leupeptin-free TCA (10µl, 2.5%w/v) to the leupeptin assay reaction mixture was determined as a necessary control.

4.2.5. Stability of Leupeptin in Conditioned Medium

In some experiments, flasks in which yolk sacs had been incubated, and from which the yolk sacs and a sample of medium had been removed, were returned to the water bath and incubated for the remainder of the experiment. A further sample of medium was then removed, for assay of its leupeptin content. Any difference between this value and the leupeptin content of the medium sampled at the time of removal of the yolk sacs would be attributable to inactivation by enzymes released from the yolk sacs into the incubation medium, ie by "conditioned" medium.

4.2.6 Recovery of Leupeptin from Yolk Sac

Known amounts of leupeptin (5-30µg) were added to aliquots (1.5ml) of a yolk-sac homogenate (one freshly-dissected yolk sac per 1.5ml of distilled water). The homogenate was then prepared for assay as described in Section 4.2.4a and 4.2.4c, ie either the homogenate was centrifuged at 1000g for 20min and the supernatant assayed for leupeptin immediately, or aqueous TCA (0.5ml, 10%w/v) was added to the whole homogenate and the TCA-soluble fraction assayed after pelleting the precipitated protein. The results were plotted as µg leupeptin detected against µg leupeptin added.

## 4.3. RESULTS

### 4.3.1. Standard Curve for BAPNA-Based Leupeptin Assay

The standard curve covered a range of leupeptin concentrations from 1-100µg/ml (ie the amount of leupeptin added in the 10µl sample ranged from 0.01 to 1.0µg.) The plot of percentage inhibition of trypsin against amount of leupeptin present (µg) is given in Fig. 4.1. The plot was approximately linear for additions of up to 0.2µg leupeptin (equivalent to 75% inhibition) then became very curved at higher inhibitor concentrations. However, when a plot of the probit value of the percentage residual activity against the log of the amount of leupeptin present was used as the standard curve, the plot was substantially linear (Fig.4.2). Linear regression analysis gave a correlation co-efficient of -0.997.

Although it was possible to detect inhibition of trypsin by very small amounts of leupeptin (ie below 0.02µg), results were not very reproducible at these concentrations. Large differences in degree of inhibition were produced by only very slightly different concentrations of leupeptin. (This could be anticipated from the steep slope of the inhibition curve, Fig. 4.1., at low leupeptin concentrations.) Conversely, at high concentrations of leupeptin (above 0.2µg in 10µl) only small differences in the degree of inhibition occurred even with fairly large changes in the amount of leupeptin present. (A change from 0.5 to 1.0µg gave a difference of only 5.4% inhibition, compared with a change from 0.01 to 0.5µg which gave a difference of 76.3%.)

Whenever possible, assays were carried out using 0.02-0.2µg leupeptin in the 10µl sample (ie 2-20µg/ml). This corresponds to a range of percentage inhibition from 20-80% (compared with 0.01-1.0µg which corresponds to 10-95% inhibition). Samples below 2µg/ml (0.02ug

in 10µl) were assayed at least three times in an attempt to minimize experimental error.

Possible sources of error in the assay method include:-

i) Errors in dispensing small volumes of reagents, especially the 10µl sample of leupeptin solution.

ii) Errors in the measurement of the rate of reaction from the chart recorder trace. Error in measurement of the gradient (in terms of mm increase along the absorbance axis per unit time) was greatest when extreme (ie very fast or very slow) rates were observed. (This occurred particularly at high concentrations of leupeptin for which the absorbance increased very little with time.) Error may also arise during estimation of the slope. Although reaction conditions were optimized to give linearity, some curvature was apparent in the uninhibited reaction, and curvature was a feature of the trace immediately after the addition of leupeptin, before enzyme-inhibitor association was complete. This effect led to a slightly subjective choice of the portion of the time-course from which the gradient was derived.

iii) Formation of small crystals of the BAPNA substrate on the sides of the plastic cuvette during the reaction. (This occurs particularly if the cuvette is scratched or unclean; Fritz et al., 1974). BAPNA is not very soluble in water (the substrate stock solution required warming to 90°C to dissolve the BAPNA) and crystals readily formed on rough surfaces, causing erratic noise in the trace. The same type of interference would be caused by cloudy reaction mixtures (caused by, for example, dust within the cuvette). Precautions were therefore taken to keep reactants and cuvettes as dust-free and as clean as possible by passing solutions through filter paper and regularly cleaning cuvettes in detergent.

### 4.3.2. Effect of Trichloroacetic Acid (TCA) on Rate of Hydrolysis of BAPNA

The standard curve was determined using leupeptin solutions in water, but many of the samples assayed for leupeptin content were solutions in 2.5% TCA. The activity of trypsin has a strong dependence on pH, therefore it was necessary to establish whether the addition of 2.5% TCA alone would affect the pH of the assay buffer and hence decrease the rate of the reaction. When 10µl of the solution of 2.5% TCA was added to the reaction mixture in place of a leupeptin sample, the rate observed did not differ from the initial rate, before the sample was added. In ten observations, the mean rate in the presence of TCA was 100.1 ± 5% (S.D.), relative to the initial rate.

### 4.3.3. Stability of Leupeptin Solutions on Storage

A preliminary investigation was carried out to determine whether samples of leupeptin dissolved in medium 199 and 2.5% TCA were stable on storage. No detectable loss of leupeptin occurred over a period of 20h at 4°C for a solution of leupeptin in medium 199 containing 10% serum and little loss occurred for a solution of leupeptin in 2.5% TCA (the amount of leupeptin decreased by 0.02µg, from 0.18 to 0.16µg, over the 20h period). It was therefore considered acceptable to store experimental samples at -20°C before assay. After storage at -20°C for several days, no decrease in leupeptin content was detectable.

### 4.3.4. Recovery of Leupeptin From Yolk-Sac Tissue

In order to determine the total amount of active leupeptin present within the yolk-sac or in a yolk-sac homogenate, it was necessary to establish how much of the leupeptin present was

detectable by the trypsin-based assay. The method is given in Section 4.2.6. Fig. 4.3. shows results obtained when either homogenate supernatants or the TCA-soluble fraction of a homogenate which contained a known quantity of leupeptin was added to the assay reaction mixture. TCA-precipitation gave complete recovery over the range of leupeptin concentrations used. However, recovery of leupeptin from the homogenate supernatant was dependent on the concentration of leupeptin added to the homogenate. At low concentrations recovery was rather poor, at about 0.64µg leupeptin/mg yolk-sac protein only 35% of the added leupeptin could be detected. The percentage recovery increased with increasing concentration, reaching 65% for leupeptin added at a concentration of about 2µg/mg (see Fig.4.3). In experiments in which homogenate supernatants were used to find the leupeptin content of the tissue, it was therefore necessary to use the recovery curve (Fig. 4.3) to convert the observed leupeptin content of the sample to the actual leupeptin content.

### 4.3.5 Accumulation of Active Leupeptin

Only active leupeptin could be detected by the trypsin-inhibition method, therefore these results gave values of tissue-associated leupeptin rather than uptake.

Several different incubation conditions were investiged to try to find optimal conditions for uptake so that the amount of leupeptin in the tissue would be within the detection limits of the assay method. These were with leupeptin at 50µg/ml in medium with or without 10% calf serum, and at 100µg/ml in medium without serum. Incubation periods were up to nine hours, to allow maximal tissue-association of leupeptin. The results are given in Table 4.1. For each experiment a matched control was performed, in which the uptake of $^{125}$I-PVP was measured. Uptake of $^{125}$I-PVP was linear

throughout the 9h incubation period, with a mean Endocytic Index of 2.65µl/mg/h in the absence of serum. Tissue levels of leupeptin did not increase progressively over the 9h period. The results from each experiment were very scattered. No real differences were apparent in the net uptake (µl/mg) after incubation under the different conditions of serum and leupeptin concentration. The amount of leupeptin detected in the 10µl sample of homogenate varied between 0.012 to 0.029µg (ie approximately 1-3µg/ml). These values were at the lower limit of detection, where the amount of error was greatest, (see Section 4.3.1). The values of tissue-associated leupeptin so determined are therefore not necessarily accurate.

4.3.6. Depletion of Leupeptin from the Medium

4.3.6a **During Incubation** The concentration of leupeptin in the medium at the start of an uptake experiment was compared with that after various periods of incubation with yolk sacs. No general trend of loss of leupeptin with increased incubation time was apparent. Likewise, little change was observed in the concentration of radioactivity (c.p.m.) in the $^{125}$I-PVP control medium over the same incubation period, indicating that medium depletion through uptake and retention within the yolk sac would not be detectable if uptake of leupeptin occurred by fluid-phase pinocytosis alone. (A rate of uptake of 2.6µg/mg/h, as observed for $^{125}$I-PVP, would lead to a loss of 4% of the substrate from the medium via uptake and retention in the yolk sac. If uptake of leupeptin occurred at the same rate as fluid-phase pinocytosis, a loss of only 2 or 4µg leupeptin ie 0.3-0.6µg/ml, for medium concentrations of 50 and 100µg/ml respectively, would be expected. This would not be detectable by the assay.)

4.3.6.b **In Conditioned Medium** The stability of leupeptin in conditioned medium at 37°C was measured by comparing the amount of leupeptin in the medium at the time of removal of the yolk sacs with that observed up to 7h later. If any loss of activity occurred leupeptin may have been partially/fully degraded and/or inactivated by enzymes that had been released from the yolk-sac tissue. However, no such loss of leupeptin was observed. (Indeed in many samples the amount of inhibitory activity in the medium appeared to increase with time. This must reflect error in the assay.)

4.3.7. Optimal pH for Inactivation of Leupeptin by Yolk-Sac Homogenate

The amount of leupeptin detectable after incubation at various pH's with a yolk-sac homogenate was compared with that remaining after incubation at the same pH for the same period, but in buffer alone. TCA was added to both homogenate and buffer incubation mixtures before assay of the remaining leupeptin content. The difference between these values was a measure of the amount of homogenate-mediated leupeptin inactivation that occurred at each particular pH. The amount of leupeptin remaining at each pH in the absence of homogenate was a measure of the stability of leupeptin at that pH and formed a necessary control experiment.

Figure 4.4. shows the stability of leupeptin over the pH range 3.5 to 9.7 in homogenate-free buffer solutions. The amount of leupeptin remaining decreased gradually on increasing the pH from 3.5 to 8.5, by which pH only about 70% of the leupeptin added remained after incubation. After incubation at pH 9.5 the activity of leupeptin was greatly reduced, therefore homogenate-mediated inactivation at this pH was not investigated.

The amount of homogenate-mediated inactivation at each pH,

expressed as µg leupeptin inactivated per mg homogenate-protein per hour, is shown in Fig. 4.5. The rate of inactivation at each pH for the different experiments varied considerably. This may have been caused by differences in the activity of enzymes in each individual homogenate. In one experiment, mercaptoethanol was included in the incubation mixture. Mercaptoethanol provides free thiol groups, which are essential for the activity of cysteine proteinases, therefore it was thought that the presence of excess of this cofactor may lead to an increased enzyme activity and possibly increased leupeptin inactivation. However, results show that less inactivation occurred compared with results obtained using a similar concentration of homogenate protein, suggesting that free thiol groups are not an essential cofactor and may even prevent leupeptin inactivation. In most experiments, the amount of leupeptin inactivated did not change much with pH, which suggests that leupeptin inactivation occurred over a wide range of pH. Either the enzyme responsible had a very broad pH optimum or a number of enzymes, with different pH optima but similar kinetic characteristics, were responsible for inactivation.

An alternative possibility was that the buffering capacity of the dilute buffers used was insufficient to maintain the desired pH over the incubation period. The pH profile of degradation of $^{125}$I-BSA$_{fd}$ was measured to check for differences in proteolytic enzyme activity in buffers of different pH. Degradation of $^{125}$I-BSA$_{fd}$ is known to have a sharp pH optimum at pH 4 (Livesey & Williams, 1979). The pH profile of $^{125}$I-BSA$_{fd}$ degradation ~~together with matched leupeptin inactivation results, obtained using the same conditions of buffer, homogenate and incubation time,~~ is shown in Fig 4.6. The degradation of $^{125}$I-BSA$_{fd}$ showed a pH optimum at 4.5, even though inactivation of leupeptin in the same buffers did not have any obvious pH dependence. This suggests that the buffers

maintained the expected pH throughout the experiment, and that the yolk-sac enzymes were still active on homogenization and incubation at 37°C.

4.3.8. Time-Course of Inactivation of Leupeptin by Yolk-Sac Homogenate

Aliquots of homogenate in phosphate buffer, pH 7.4, were incubated with either 20 or 50µg leupeptin. At various intervals between 2 and 30h, TCA was added to one aliquot and the TCA-soluble fraction assayed for leupeptin. The amount of leupeptin remaining was subtracted from that added to find the amount of leupeptin inactivated. This was expressed as µg/mg yolk-sac protein. Fig. 4.7 shows a graph of the µg leupeptin inactivated per mg yolk-sac protein against time (h).

Homogenate-mediated inactivation of leupeptin occurred in all experiments. However, the rate of inactivation differed greatly between experiments. Several differences in experimental conditions were used. The homogenate preparations were different for each experiment: the homogenate in experiment 1 was used fresh, whereas those used for experiment 2 and 3 had been stored at -20°C for 5 and 1 week(s) respectively. Also 50µg of leupeptin was added to the homogenate in experiment 1, compared with 20µg in experiments 2 and 3. The maximum rate of inactivation, observed in experiment 1, was approximately 0.2µg/mg protein/h. Only comparatively slight increases in the amount of leupeptin inactivated were observed over the 22h incubation period in experiments 2 and 3.

The effect of variation of protein concentration on the rate of inactivation was determined. The same homogenate was used for all incubations, so that the effects of inter-homogenate variation were avoided. Different concentrations were achieved by diluting the

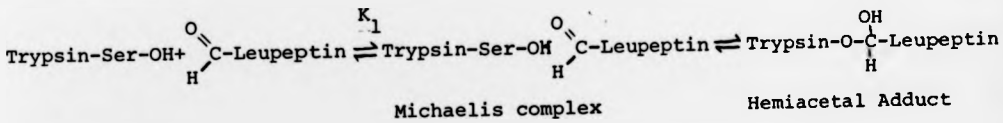
homogenate with distilled water. 50µg of leupeptin was added to each aliquot of diluted homogenate, and the reaction stopped after 20h incubation at 37°C. The results, expressed as µg leupeptin inactivated per h against mg yolk-sac protein per h are shown in Fig. 4.8. The increase in inactivation with increasing protein concentration indicates that inactivation was homogenate-mediated. The graph is non-linear, which suggests that the rates of inactivation observed in the previous experiment, in which different homogenate protein concentrations were used, cannot be compared.

## 4.4. DISCUSSION

### 4.4.1. Inhibition of Trypsin Catalysed Hydrolysis of BAPNA as a Method of Detection of Leupeptin

Enzyme inhibition assays have been used for many years to estimate the amount of inhibitor in a system. Several important conditions must be met if such assays are to be strictly quantitative. Many of these relate to the stability of the enzyme-inhibitor complex.

The interaction between leupeptin and trypsin is reversible, even though a covalent bond forms between the two. Kuramochi et al. (1979) propsed a mechanism in which the association of leupeptin with trypsin occurs in two steps; first a non-covalent Michaelis complex forms, then a hemiacetal adduct:-




The dissociation constant for the formation of the Michaelis complex ($K_1$) was $1.24 \times 10^{-3}$M which implies a fairly low affinity between inhibitor and enzyme. The overall dissociation constant ($K_D$) for the association of leupeptin with trypsin was found to be about $2 \times 10^{-8}$M at pH 8.2.

Conditions necessary for the quantitative analysis of inhibitors have been described by Bergmeyer (1984), and are briefly summarized below.

Ideally a linear relationship should exist between the degree of inhibition observed and the amount of inhibitor added. (This reflects a stoichiometric interaction between enzyme and inhibitor.) However, all enzyme-inhibitor interactions are non-linear at high inhibitor

concentrations; linearity may cease anywhere between 70 and 95% inhibition. Preferably only the linear part of an enzyme-inhibitor interaction curve is used for calculation of the amount of inhibitor present. A linear relationship is not so important when an experimentally-determined standard curve is used for the calculation of the amount (µg) of inhibitor present. Any non-linearity in the interaction is apparent in the standard curve and is therefore taken into account when calculating the amount of inhibitor present. For the interaction between leupeptin and trypsin, the inhibition curve was non-linear beyond 75% inhibition, but the probit transformation of the standard curve was essentially linear over the whole range.

Another important consideration in any inhibitor assay is competition between the inhibitor and substrate for the active site of the enzyme. The interaction between inhibitor and enzyme should have a $K_i$ very much lower than the $K_m$ for the interaction between substrate and enzyme. If the $K_i$ is $10^3$ to $10^4$-fold smaller than the $K_m$, competition can be ignored as effectively all the inhibitor will be bound to the enzyme. (An alternative means of overcoming any competition would be to set the enzyme concentration $10^2$ to $10^3$-fold higher than the $K_i$ of the inhibitor. However, this would have the attendant disadvantage of making the assay less sensitive to changes in inhibitor concentration.) The dissociation constant of the leupeptin-trypsin complex is $2 \times 10^{-8}$M (Kuramachi et al., 1979) whereas the substrate BAPNA has a $K_m$ of $4.3 \times 10^{-4}$M (Fritz et al., 1974), therefore the difference in affinity for the enzyme should overcome competition. (The initial association reaction of leupeptin with trypsin has a $K_i$ of $1.24 \times 10^{-3}$M, therefore competition may occur initially, but the subsequent reaction of leupeptin to form a covalently-linked complex should ensure all inhibitor becomes bound to the enzyme.)

The spectrophotometric method of leupeptin detection obeyed all the necessary conditions for inhibitor assays, therefore this assay was considered to be a potential method of determining quantitatively the leupeptin content of samples.

4.4.2. Estimation of Leupeptin Content of Media, Homogenates, etc

The standard curve was determined using leupeptin dissolved in distilled water, therefore any change in sample composition had to be checked for its effects on the enzyme assay.

**4.4.2a. TCA** Results in Section 4.3.2. showed no change in the rate of BAPNA hydrolysis; the triethanolamine buffer used for the assay appeared to maintain the optimum pH even after addition of the small volume of TCA solution.

**4.4.2b. Medium 199** Phenol red, which is present in medium 199, has an absorbance peak at 557nm for pH greater than 7. Addition of 10µl of leupeptin-free, diluted medium 199 to the assay mixture did not affect the observed absorbance (at 405nm), or the rate of reaction.

**4.4.2c Homogenate** Addition of tissue homogenate (centrifuged to remove cell debris) was found to slightly decrease the observed rate of BAPNA hydrolysis. The rate was decreased to about 91% ± 3 relative to the rate before any addition, corresponding to about 0.01 µg of leupeptin. This value was subtracted from any results from experiments in which homogenate that contained leupeptin was added to the assay system. The apparent decrease in rate of hydrolysis of BAPNA may have been caused by an increased turbidity of the assay solution (Fritz et al., 1974) or could have been caused by the presence of trypsin inhibitors within yolk-sac tissue. (To date no such inhibitors have been reported.)

Because the substrate BAPNA is not specific for trypsin, and can

be hydrolsed by enzymes likely to be present in the homogenate (eg cathepsin B), an increased rate of reaction after addition of the homogenate to the assay might be expected. However, no such increase was observed, either because the amount of homogenate enzymes was small in comparison to the amount of trypsin present in the reaction mixture (5µg) or because their pH optima were far lower than the assay pH of 7.8.

The recovery of leupeptin from a yolk-sac homogenate depended on how the homogenate was prepared for assay. Addition of the centrifuged homogenate directly to the assay reaction mixture resulted in a low recovery of leupeptin. However, when the homogente was precipitated with TCA total recovery was achieved; all leupeptin that had been added to the homogenate was detected in the TCA-soluble fraction. Clearly the untreated homogenate caused loss of leupeptin, by a mechanism which was not active in the presence of TCA. Possible mechanisms are described below.

i) Leupeptin binds tightly to its 'target' enzymes, some of which would be present in the homogenate (eg cathepsins B,(H), and L). Leupeptin bound to homogenate enzymes would not be detected in the assay, since leupeptin must bind to trypsin to be detectable. The proportion of leupeptin bound to homogenate enzymes or trypsin would depend on the $K_i$ of leupeptin for each enzyme at a pH of 7.8.

ii) Another possible sequestor of leupeptin could be the pelleted cell debris, which was not assayed. Leupeptin could become occluded by mechanical trapping within the pellet, or by binding to membranes, connective tissue, and other pelleted material. Since TCA-precipitation gave complete recovery, leupeptin must not become trapped in a pellet of denatured proteins, therefore mechanical trapping would probably not account for all of the sequestration of leupeptin.

4.4.3. Tissue Accumulation of Leupeptin

The amount of leupeptin associated with the tissue was barely detectable by the BAPNA assay. The experimental values fell in the region of the standard curve that corresponds to high probit values, for which errors were relatively large. The incomplete recovery of leupeptin from the yolk-sac homogenate introduced additional sources of error, both in the conversion of the amount of leupeptin observed to the amount estimated to be present in the yolk sac, and by further lowering the amount of leupeptin available for detection. Thus only overall trends in the results rather than reliable quantitative data are available for discussion.

Firstly, there was no detectable progressive increase in the quantity of leupeptin that became associated with tissue on incubation with leupeptin. This lack of accumulation could suggest that there is loss of leupeptin from the tissue after uptake, since uptake of $^{125}$I-PVP continued progressively over the 9h incubation period. (Loss of leupeptin from the tissue could be caused either by inactivation of the inhibitor within yolk sacs, or by release of intact leupeptin from yolk sacs.)

Secondly, there was no detectable difference in the quantity of leupeptin associated with yolk-sac tissue that had been incubated with or without serum or at a leupeptin concentration of 50 or 100µg/ml of medium (Table 4.1). However, the degree of scatter of results was such that no definite conclusions could be drawn.

The minimal rate of uptake of leupeptin would be that of fluid-phase pinocytosis. Uptake via this route would be greatest at the higher concentration of leupeptin (100µg/ml) when serum was absent (since the rate of pinocytosis of $^{125}$I-PVP has been shown to be decreased in the presence of 10% serum; Forster & Williams, 1984). The rate of fluid-phase pinocytosis, given by the rate of uptake of

the marker $^{125}$I-PVP, is about about 3µl/mg/h in serum-free medium. Hence, assuming no loss from the tissue after uptake, a typical yolk-sac sample of 12mg protein would take up about 3µg of leupeptin by fluid-phase pinocytosis after 8h incubation in serum-free medium with leupeptin at a concentration of 100 µg/ml. This should give a concentration of leupeptin in the tissue homogenate of about 2µg/ml, which is only just within the detection limit. Hence, if uptake of leupeptin occurred only by fluid-phase pinocytosis, the time-course of accumulation over 3-9h would be very difficult to quantitate.

Overall, the results suggest that leupeptin associated with the tissue only slowly, and is possibly lost from the tissue after uptake.

### 4.4.4. Loss of Leupeptin from the Incubation Medium

Any progressive decrease in the amount of leupeptin in the incubation medium during incubation with yolk sacs would have serious implications on any attempts to quantitate accumulation by the tissue.

However, no overall decrease in the amount of leupeptin in the medium (either in the presence or absence of yolk-sac tissue) was observed. The lack of depletion from the medium indicates that leupeptin is stable at 37°C in medium 199 for up to 9h, and that it is not taken up rapidly and retained or degraded within the tissue (c.f. $^{125}$I-BSA$_{fd}$, Williams *et al.*, 1975b). Leupeptin must be resistant to inactivation by enzymes associated with the outer face of the plasma-membrane and by enzymes released into the bulk medium, (unlike the longer peptides: calcitonin, glucagon, insulin, and insulin B-chain; Livesey, 1978). Protection from degradation may be due to its action as an inhibitor of any such enzymes (in which case leupeptin would show an affinity for cell membranes) or because the

blocked amino end and the aldehyde moiety at the carboxyl end lead to little affinity for such enzymes, and hence make it a poor substrate.

### 4.4.5. Inactivation of Leupeptin by a Yolk-Sac Homeqenate

The experiments described in Sections 4.3.7. and 4.3.8 show that enzymes present in a yolk-sac homogenate are capable of inactivating leupeptin. The mechanism of inactivation (eq total degradation, oxidation/reduction of the essential aldehyde group) and the enzyme or enzymes responsible could not be identified from these results.

Many reports suggest circumstanial evidence to indicate that leupeptin may become inactivated , *in vitro* and *in vivo*. Effects on intact cells tend to be reversible over 24h after removal of leupeptin from the system, suggesting slow inactivation of leupeptin may occur within cells (although slow leakage from the cell or re-synthesis of inhibited proteases could also account for such observations). For example, autophagolysosomes, which accumulate in liver cells after administration of leupeptin, do not persist for longer than 24h (Furuno *et al.*, 1982a; Henell & Glaumann, 1984; Ishikawa *et al.*, 1983). Also Dunn & Aronson (1977) found that protein degradation was not inhibited when leupeptin was administered 24h before measurement of degradation began, and Knowles *et al.*(1981) suggested that the lack of progressive increase in degree of inhibition of proteolysis caused by leupeptin in yolk-sac tissue may be due to lack of accumulation of the inhibitor as a result of inactivation.

More direct evidence of inactivation of leupeptin is given by the work of R.J. Beynon and co-workers. Beynon *et al.*(1981) showed that leupeptin and chymostatin (another proteinase inhibitor derived from *Streptomyces*) were inactivated by mouse-liver homogenates. Some of the characterisics of the enzyme or enzymes responsible were

established by carrying out inhibitor studies and by determining the pH profile of inactivation. Later (Brown & Beynon, 1983) it was shown that homogenates of many other tissues were capable of inactivating leupeptin. In order of decreasing 'leupeptinase' activity, tissues tested were:- gut mucosa, spleen, gut smooth muscle, lung, kidney, brain, heart, liver and skeletal muscle. For mice, the total body capacity for inactivation was calculated as 750µg/24h. The subcellular distribution showed maximum activity in the cytosol, but high levels of activity were apparent in the microsomal and heavy particulate fractions. Place et al. (poster communication) isolated from rat liver two enzymes capable of inactivating leupeptin, and characterized one of these. This enzyme appeared to be a metallo-enzyme, that hydrolysed the Leu-Arginal bond. A total liver capacity for inactivation was propsed to be 8mg leupeptin per hour at 37°C, pH 7.5. Other evidence of inactivation is given by Tanaka (1983), who showed that leupeptin was metabolized after administration to rats either orally or intraveneously.

The finding that leupeptin was inactivated by a yolk-sac homogenate was therefore not unexpected. Attempts to further characterize the conditions that gave optimal inactivation proved inconclusive. The rate of inactivation differed greatly between experiments in which different homogenates were used, even though the method of preparation of the homogenate was similar. The amount of inactivation that occurred was shown to depend in a non-linear fashion on the amount of homogenate protein present, so preventing comparison of rates of inactivation in homogenates of different protein content. Furthermore, the rate of inactivation appeared to depend on the amount of leupeptin added initially, being greater for 50µg than for 20µg of leupeptin (Fig. 4.7).

Large differences in the rate of inactivation were also apparent

in experiments in which the pH profile of inactivation was studied. The low activity of the homogenate containing mercaptoethanol supports the results of Beynon et al. (1981), who also found that 'leupeptinase' activity was inhibited by thiol agents. However, unlike the results reported in that paper, no pH optimum was observed for inactivation of leupeptin by a yolk-sac homogenate. Possibly more than one enzyme in the yolk-sac homogenate was capable of inactivating leupeptin, each having a maximum rate at a different pH, or the enzyme capable of leupeptin inactivation was active over a broad pH range. $^{125}I\text{-}BSA_{fd}$ was degraded, as expected, with a pH optimum of about 4, indicating degradation of this substrate was brought about by lysosomal enzymes, which were active under the conditions of assay.

Leupeptin was shown to lose activity on incubation in buffers of different pH, the rate of inactivation being greatest at pH 9.5. Possibly part of the structure essential for activity, namely the L configuration and aldehyde moiety of arginal, became changed at high pH. Racemization of the chiral centre of arginal was reported to occur slowly at room temperature at pH 8.0; 50% activity, ie total racemization, being achieved after 4 days (Umezawa & Aoyagi; 1977). Aldehyde groups are highly reactive and can undergo a variety of acid or base-catalysed reactions, eg. aldol condensation.

To summarise:-

This method of leupeptin assay was useful in obtaining a general impression of the uptake characterstics of leupeptin into yolk-sac tissue, but was insufficiently sensitive to permit quantitation of the small amounts of leupeptin associated with the tissue. The fact that only small amounts were observed within the tissue suggests slow uptake and/or rapid loss of leupeptin.

Inactivation of leupeptin by yolk-sac homogenates also occurred, but no details of rate could be measured due to large variations between experiments. None of the homogenates showed any marked pH optimum for leupeptin degradation.

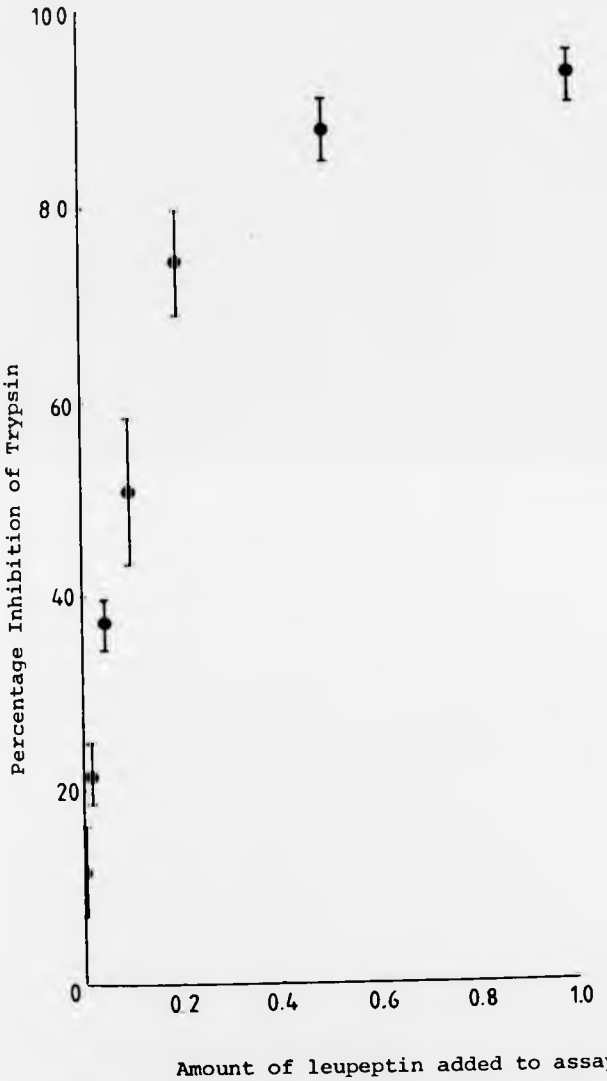



Fig. 4.1 Inhibition Curve for Trypsin and Leupeptin using BAPNA for the Substrate

The percentage of enzyme activity that remained when various amounts of leupeptin were added to a fixed amount of trypsin was determined as described in Section 4.2.1. The points represent the mean percentage inhibition (± standard deviation).

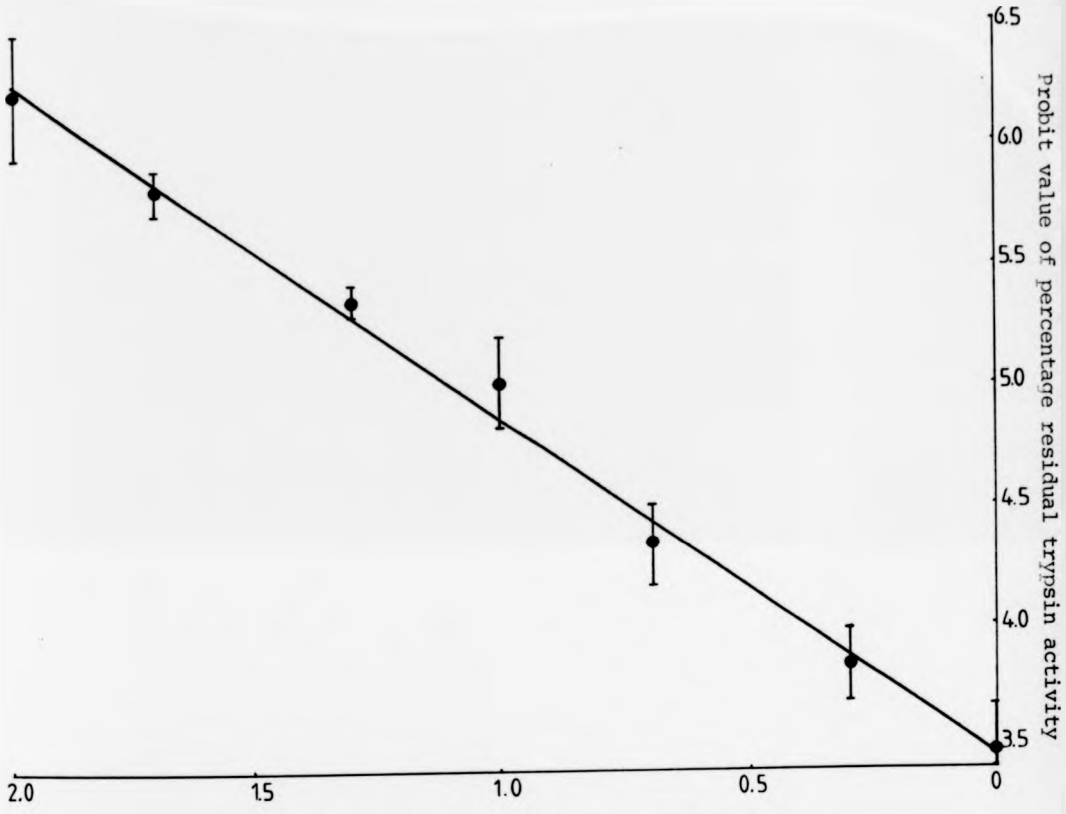



Fig. 4.2 Probit Transformation of the Inhibition Curve for Trypsin and Leupeptin: Standard Curve

The percentage of trypsin activity that remained in the presence of various amounts of leupeptin (determined as described in Section 4.2.2) was converted to its probit value using tables. The mean probit values (± standard deviation) then plotted against the logarithm of each amount of leupeptin present to obtain a linear standard curve

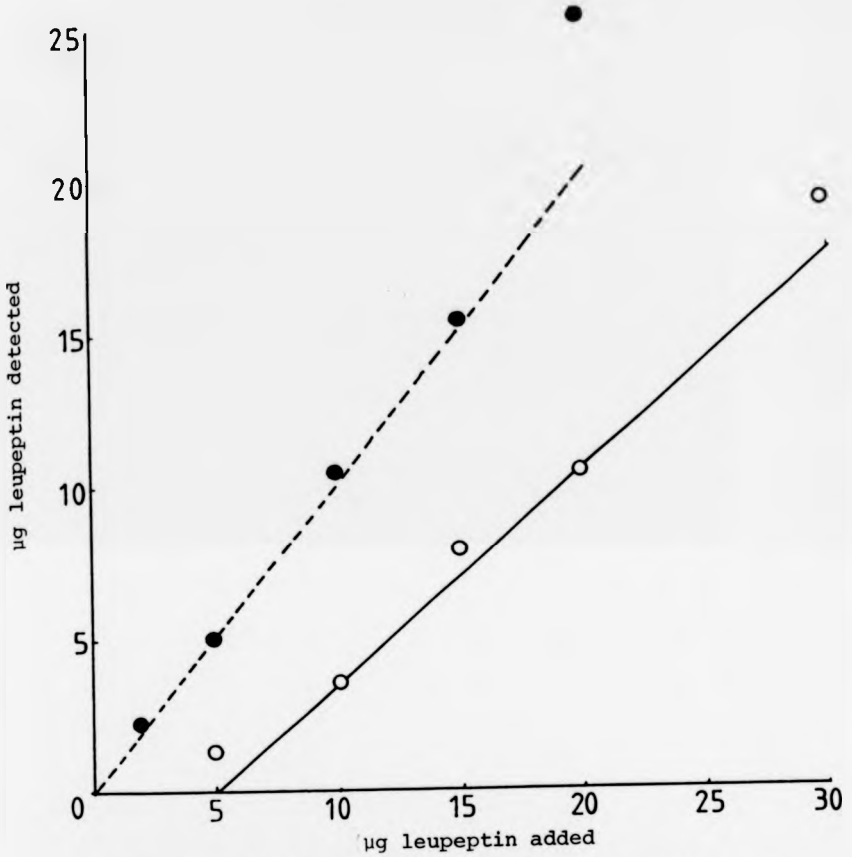



Fig. 4.3 Recovery of Leupeptin from Yolk Sacs

Yolk sacs were prepared for assay of leupeptin content as described in Section 4.2.6. The graph shows the mean quantity of leupeptin detected in the homogenate and TCA-solubles against the amount added to the yolk-sac tissue

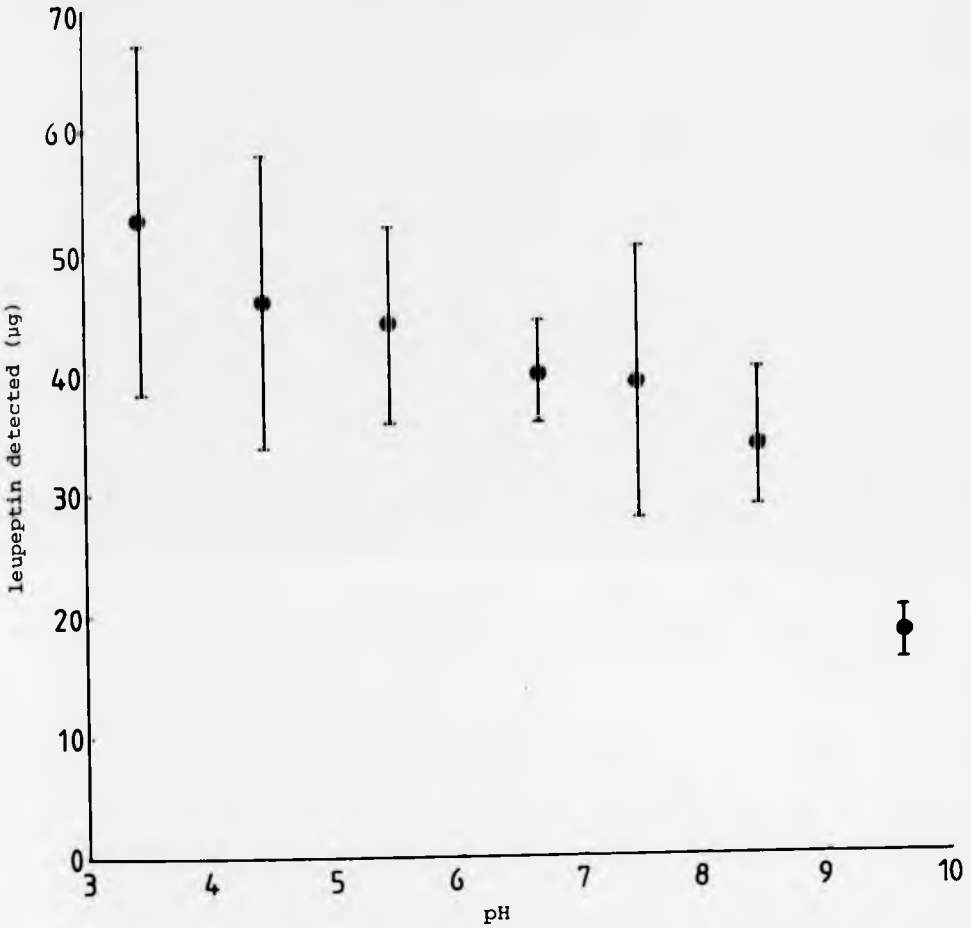



Fig. 4.4 Stability of Leupeptin in Buffers of pH 3.5-9.5

Leupeptin (50 µg) was incubated with buffers for 15.5h as described in Section 4.2.3. The amount of leupeptin that remained after incubation was determined as described in Section 4.2.1. The graph shows mean values ± standard deviation at each pH.

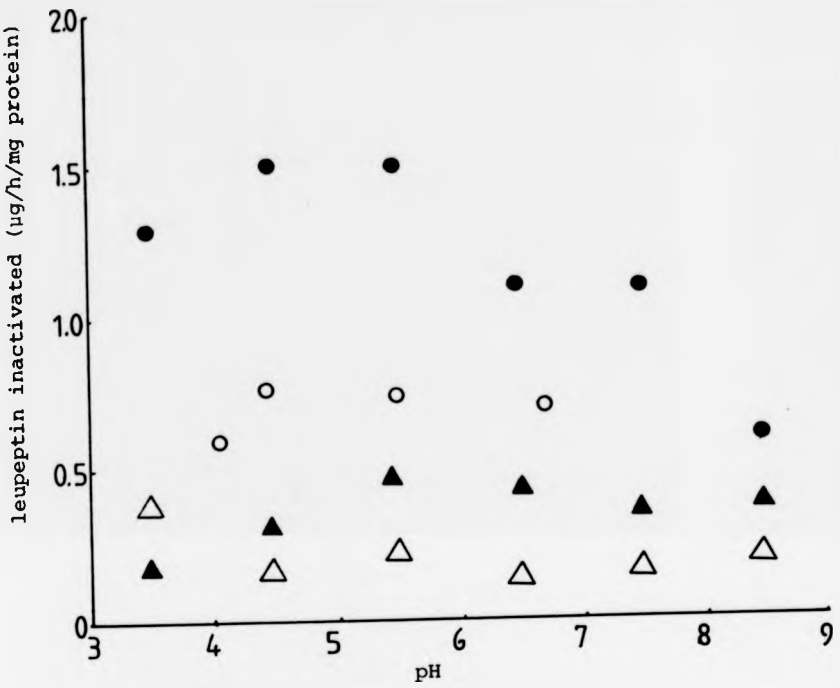



Fig. 4.5 Inactivation of Leupeptin by Yolk Sac Homogenates over the pH range 3.5-8.5

Leupeptin (50 µg) was added to yolk sac homogenate mixed with buffers, as described in Section 4.2.3. The amount of leupeptin that remained after incubation was determined as described in Section 4.2.1. The amount of homogenate-mediated leupeptin inactivation was calculated from the difference between the amount of leupeptin remaining after incubation in the presence and absence of homogenate protein, and expressed as µg inactivated per mg protein per hour. Each plot represents results from a single experiment, carried out under similar conditions.

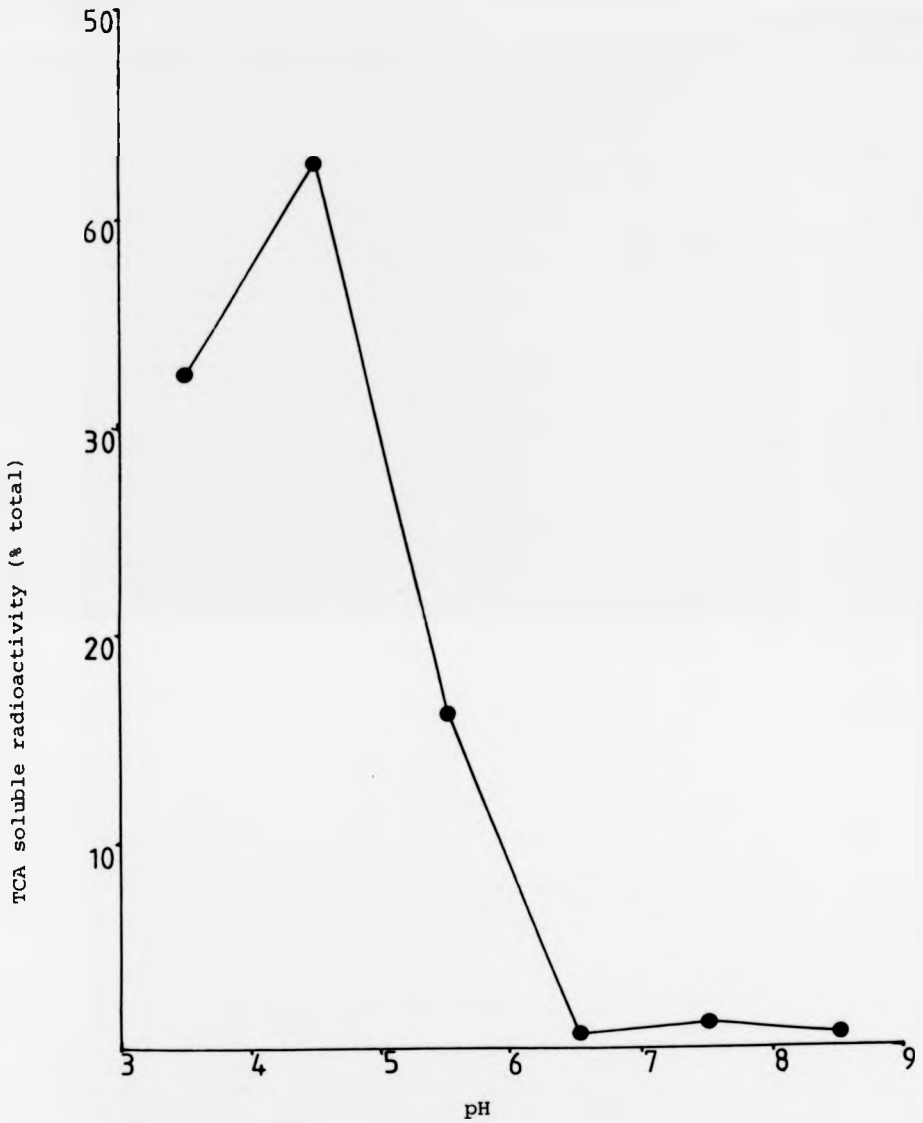
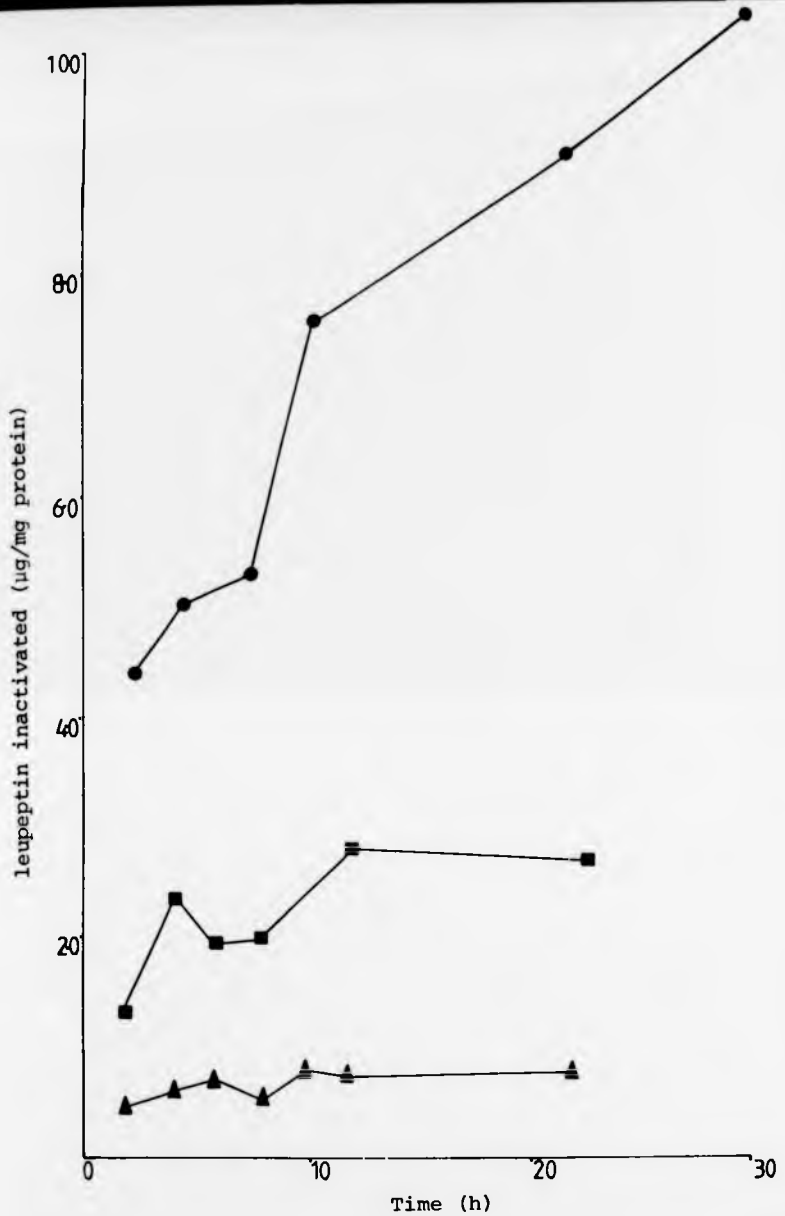



Fig. 4.6 Degradation of $^{125}$I-BSA$_{fd}$ by Yolk Sac Homogenate over the pH range 3.5-9.7

$^{125}$I-BSA$_{fd}$ (approx. 50 µg) was incubated with yolk-sac homogenate mixed with the buffers described in Section 4.2.3 and incubated at 37°C for 15-16h. The percentage of $^{125}$I-BSA$_{fd}$ that was degraded at each pH was determined from the percentage of TCA-soluble radioactivity present at the end of incubation.




● Exp. 1. Protein 4.2 mg/ml; Leupeptin 50 µg; fresh tissue

■ Exp. 2. Protein 20 mg/ml; Leupeptin 20 µg; tissue frozen 5 week

▲ Exp. 3. Protein 9.7 mg/ml; Leupeptin 20 µg; tissue frozen 1 wee

Fig. 4.7 Time-Course of Inactivation of Leupeptin by Yolk-Sac Homogenates

Leupeptin was incubated with yolk-sac homogenates as indicated. At various intervals TCA was added and the samples assayed for leupeptin as described in Section 4.2.1. The amount inactivated was calculated from the difference between the amount detected and the amount added, and expressed as µg leupeptin inactivated per mg protein. Each plot represents a separate experiment using slightly different incubation conditions.

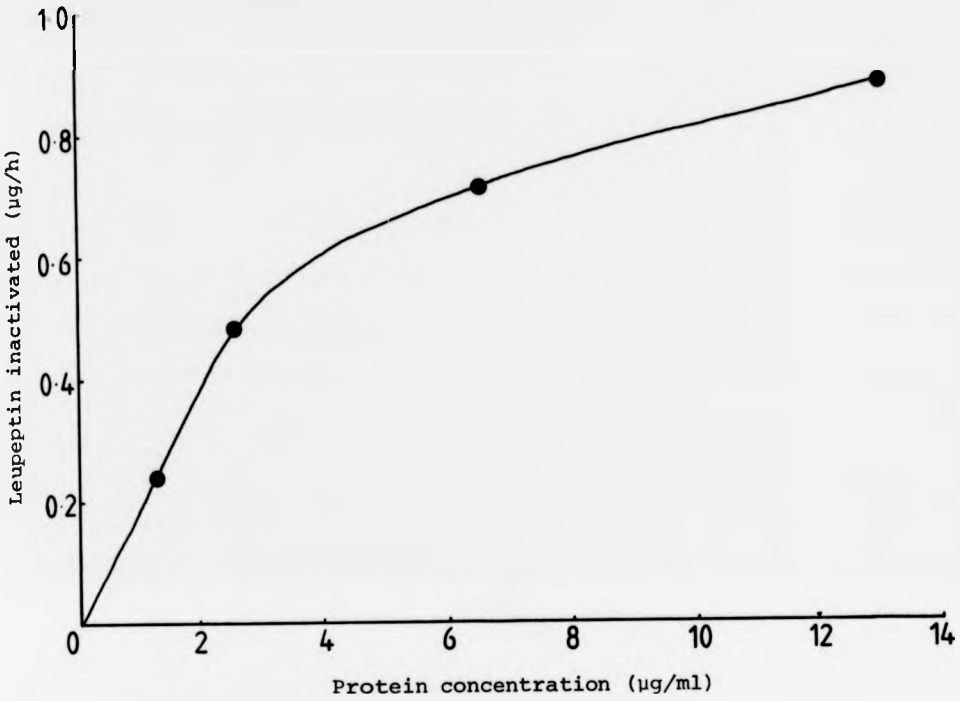



Fig. 4.8 Inactivation of Leupeptin by Different Concentrations of Yolk-Sac Homogenate Protein

A yolk-sac homogenate was diluted with phosphate buffer pH 7.5. Leupeptin (50 µg) was incubated with samples of the diluted homogenate for 15.5h, then TCA (final concentration 2.5% w/v) added. The amount of leupeptin inactivated, calculated from the difference between the amount detected after incubation and the amount added was expressed as µg leupeptin inactivated per h, and plotted against mg protein present.

Table 4.1

Accumulation of active leupeptin and $^{125}$I-PVP

The accumulation of active leupeptin under various incubation conditions was determined as described in Section 4.2.2. Uptake of $^{125}$I-PVP was monitored under the same conditions, as a control. Results shown are from individual experiments.

| Experiment | | I | | II | | III | |
|---|---|---|---|---|---|---|---|
| Incubation Conditions | | 10% calf serum Leupeptin at 50 µg/ml | | 0% calf serum Leupeptin at 50 µg/ml | | 0% calf serum Leupeptin at 100 µg/ml | |
| Substrate | | Leupeptin | $^{125}$I-PVP | Leupeptin | $^{125}$I-PVP | Leupeptin | $^{125}$I-PVP |
| Accumulation (µl/mg protein) after incubation period | 3h | 3.3 | 6.0 | 3.1 | 8.4 | 5.5 | 11.4 |
| | 5h | 5.3 | 11.7 | 3.5 | 11.3 | 3.0 | 14.4 |
| | 7h | 3.6 | 15.7 | 3.9 | 14.7 | 3.7 | 17.5 |
| | 9h | 2.9 | 26.7 | 3.6 | 18.5 | 1.7 | 26.9 |
| Endocytic Index | | | 3.25 | | 1.69 | | 2.48 |

# CHAPTER 5

## FLUORIMETRY METHOD OF LEUPEPTIN DETECTION

## 5.1. INTRODUCTION

The main aims of the experiments described in this chapter were essentially the same as those of Chapter 4, ie to compare the accumulation kinetics of leupeptin with those of markers, whose mode of uptake and intracellular fate are known, in order to establish the mechanism of uptake and the intracellular fate of leupeptin.

The results obtained using the BAPNA-based assay described in Chapter 4 suggested that the quantity of active leupeptin within the tissue was low and did not increase over a 3-9h incubation period. The small amounts of leupeptin within the tissue were impossible to assay accurately because the assay was not sufficiently sensitive. Thus little information could be obtained regarding the kinetics of leupeptin accumulation, preventing the suggestion of a possible mode of uptake of leupeptin.

The work reported in the present chapter used a more sensitive, method of leupeptin detection for which a fluorogenic substrate, carbobenzoxy-phenylalanyl-arginyl-7-aminomethylcoumarin (Z-Phe-Arg-AMC), was used instead of BAPNA as the trypsin substrate.

A major advantage of fluorogenic substrates over chromogenic substrates is that, in general, it is possible to determine fluorescence with greater sensitivity than absorbance. [In fluorimetry the signal measured is a increase from zero intensity, when light is emitted during excitation, whereas for chromogenic measurements the absorbance signal is related to the ratio of intensity of incident and transmitted light (Allinger et al., 1976). This change in intensity will be small relative to the overall intensity of incident and transmitted light. For any measurement a small increase from zero is generally determined more accurately than a small decrease from a large 'standing' values.]

For trypsin assays, the AMC leaving group has an added advantage

over the commonly-used chromophore benzoyl-Arg-nitroanilide (BAPNA) because it has been shown by Kanaoka et al. (1977) that the amide link in benzoyl-Arg-aminomethylcoumarin is more susceptible to cleavage by trypsin than is that of BAPNA.

Aminomethylcoumarin substrates also have many advantages over traditional fluorogenic substrates such as naphthylamides. 7-Amino-4-methylcoumarin (AMC) is very highly fluorescent and can be detected at very low concentrations. (The maximum fluorescence intensity of AMC is approximately 14 times greater than that of naphthylamine). The fluorescence intensity of AMC does not change with pH over the range 4 to 7, whereas that of naphthylamine shows a strong pH dependence. No toxic effects have been reported for AMC, unlike naphthylamine which is classed as a potent carcinogen. Although aminomethylcoumarin substrates are, at present, more expensive than corresponding naphthylamine or chromogenic substrates, the high cost is offset by the very small amounts required for detection in each assay.

Because very low concentrations of AMC can be measured, less trypsin was required in each assay to produce detectable amounts of AMC from Z-Phe-Arg-AMC. Hence the amount of leupeptin required to produce a measurable inhibition of the enzyme was also lowered (Fritz et al., 1974).

It was hoped that the decreased detection limit for leupeptin would enable the small amounts of leupeptin within tissue to be measured accurately. The amount of leupeptin within yolk-sac tissue after shorter periods of incubation with leupeptin could then be measured, to determine the initial rate of accumulation of intact leupeptin. The rate of accumulation was compared with the rate of uptake of $^{125}$I-PVP to assess whether uptake occurred by fluid-phase pinocytosis. Further evidence was obtained by using an inhibitor of

pinocytosis (ammonium chloride, 20mM) to establish whether accumulation of leupeptin was blocked.

Possible intracellular fate(s) of leupeptin were followed by measuring the loss of leupeptin from yolk sacs using a modification of the method originally described by Williams et al. (1975a). The standard method of measuring loss of a substrate from pre-loaded tissue is to monitor the appearance of the substrate in fresh substrate-free medium. However, in such experiments, only release of intact leupeptin would be detected; it would not be possible to monitor inactivation within the tissue followed by release of degradation products. Consequently, loss was followed by monitoring the amount of active leupeptin remaining within tissue that had been loaded with leupeptin, after various periods of re-incubation.

It was hoped to distinguish between loss of leupeptin activity occurring via lysosomal inactivation and via release of intact leupeptin, by assaying the re-incubation medium for leupeptin, and by using ammonium chloride (10mM) to inhibit proteolysis within the yolk sac. The effect of ammonium chloride on the loss of inhibitory leupeptin from yolk sacs was therefore determined, and compared with its effect on both the release of $^{125}$I-PVP (which only occurs via exocytosis or cell death, Williams et al., 1975a) and the degradation of $^{125}$I-BSA$_{fd}$.

To summarise, the following were investigated using the Z-Phe-Arg-AMC-based leupeptin assay:-

i) The rate of accumulation of active leupeptin in yolk-sac tissue was compared with the rate of uptake of $^{125}$I-PVP.

ii) The effect of ammonium chloride (20mM) on the accumulation of leupeptin (and $^{125}$I-PVP) was investigated.

iii) The rates of loss of leupeptin, $^{125}$I-BSA$_{fd}$, and $^{125}$I-PVP were measured, and compared with the rate of loss determined in the presence of ammonium chloride.

## 5.2. MATERIALS AND METHODS

### I. MATERIALS

**Equipment**

| | |
|---|---|
| Fluorimeter | Model 3000 Fluorescence Spectrophotometer. Perkin-Elmer, Beaconsfield, Berks, U.K. Excitation wavelength 360 nm, emission wavelength 460 nm. |
| Chart Recorder | Model 056-1002 Hitachi Ltd. Tokyo, Japan. |

**Reagents**

| | |
|---|---|
| Z-Phe-Arg-7-AMC | Code PB 13520. Cambridge Research Biochemicals Ltd., Cambridge. Z-Phe-Arg-7-AMC was dissolved in DMSO (3mg/ml, 4.75mM) to produce a stock solution, stored at -20°C. Stock solution was diluted 10µl:2.5ml (to 12µg/ml, 19µM) in HEPPS buffer before use. |
| HEPPS Buffer | 0.1M HEPPS, dissolved in distilled water, adjusted to pH 8.0 with NaOH (1M). |
| Trypsin | 0.1mg/ml, dissolved in HCl acidified water (pH 3). |
| Leupeptin | 1mg/ml stock solution, dissolved in water. Added to yolk-sac incubations or diluted as necessary for use as standards in assay. |

$^{125}I\text{-}BSA_{fd}$ Prepared as described in Section 2.1. A stock solution of about 100µg/ml in medium 199 was prepared.

## II. METHODS

### 5.2.1. Carbobenzoxy-Phenylalanyl-Arginyl-7-Aminomethylcoumarin-Based Assay for Leupeptin

The method of assay was similar to that of the BAPNA-based assay and was suggested by Dr. R. Beynon, University of Liverpool (personal communication). The rate of trypsin-mediated hydrolysis of Z-Phe-Arg-AMC was monitored in the presence and absence of an aliquot of test solution containing leupeptin of unknown concentration. The percentage inhibition given by the leupeptin was then calculated and the corresponding concentration of leupeptin found from a standard curve of the probit of the percentage residual activity against the natural logarithm of the leupeptin concentration.

Experimental details differed slightly from the BAPNA-based assay. The amount of trypsin present in the assay was decreased by a factor of 10. Separate assay reaction mixtures were used to measure the inhibited and uninhibited rates. Trypsin and leupeptin were allowed to equilibrate together for at least two minutes before addition of the substrate, to allow complete association between the enzyme and inhibitor before initiating the reaction by addition of substrate. (This was necessary because association between enzyme and inhibitor was likely to be slower at the low concentrations used and because the hydrolysis reaction was measured over a shorter period.) The uninhibited rate was measured after a similar 'equilibration' period in the absence of leupeptin; a number of these uninhibited reaction rates were determined throughout the experimental session.

Thus the assay was executed as follows:-

HEPPS buffer (1.5ml), trypsin (10µl), and when appropriate the sample solution of leupeptin (20µl), were pipetted into a cuvette and

allowed to equilibrate for at least 2min at 25°C in a water bath. (Longer periods of equilibration did not affect the rate of reaction.) The substrate, Z-Phe-Arg-7-AMC (0.5ml), was then added, and the rate of hydrolysis monitored using the fluorimeter described in the Materials section. The rate of reaction was measured between 20 and 80 percent deflection on the chart recorder, to standardize the choice of the portion of time-course used. (Standard curve assays for leupeptin concentrations above 1µg/ml were monitored between 20 and 50% deflection only.) The percentage residual activity for each sample was calculated from the mean of the inhibited rates (experimental samples were assayed at least in duplicate; standard curve samples were assayed from 3 to 10 times) and the mean of uninhibited rates determined at the same time as inhibited samples (generally assayed about 4 times per period). The appropriate probit value of this percentage was found from tables. The concentration of leupeptin corresponding to the percentage residual activity observed for the samples of unknown leupeptin concentration was then calculated. [In later assays the amount of reactants used were decreased by half to conserve substrate, so that the volumes used were:- buffer, 0.75ml; substrate, 0.25ml; leupeptin, 10µl; and trypsin, 10µl of a 0.05mg/ml solution. The decrease in volume did not affect the standard curve.]

Other ways of plotting the standard curve data, such as computer-fitted curves, were also assessed for convenience of use.

### 5.2.2. Accumulation of Active Leupeptin

The method used to study the accumulation of active leupeptin was as described in Section 2.6. It was necessary to check whether the presence of leupeptin affected uptake of $^{125}$I-PVP when uptake of both substrates into the same yolk sac was measured. A control in

which accumulation of leupeptin and uptake of $^{125}$I-PVP was determined in separate yolk-sac incubations was performed as described in Section 4.2.2. The effect of an inhibitor of pinocytosis (ammonium chloride, 20mM) on accumulation of leupeptin was determined. Ammonium chloride (0.7ml, 200mM in distilled water) was added to medium (4.9ml) and yolk sacs incubated in this medium for at least 15min before the addition of leupeptin (0.7ml) and $^{125}$I-PVP (0.7ml) to initiate the incubation. The experiment was then continued as described in Section 2.6. In one experiment, low temperature was used to inhibit pinocytosis. The experiment was performed as described in Section 2.6 except the yolk sacs were incubated at 5°C.

5.2.3. Preparation of Samples for Leupeptin Assay

5.2.3a. **Yolk-Sac Tissue** Many different methods of preparing tissue for assay of leupeptin content were tested for suitability (eg efficiency of disruption of tissue, recovery of leupeptin and technical ease of execution). These methods are described in Appendix 2. The following method of tissue preparation was used for all experiments reported in this chapter. Yolk sacs (3 per sample) were homogenised in 0.1% Triton X-100 (1ml), in a hand-held ground-glass-on-ground-glass homogenizer. (Similar results were obtained when yolk sacs were homogenised in distilled water then the homogenate sonicated for 30sec.) The total volume of homogenate was noted. A sample of homogenate (0.5ml) was mixed with an equal volume of TCA (5% w/v), the precipitated protein separated by centrifugation (1000g, 20min), and the volume of the TCA-soluble fraction noted. This TCA-soluble fraction was used in the assay of the leupeptin content of the tissue. The remaining tissue homogenate was made up to 5ml with NaOH(1M) and assayed for protein content and for radioactivity as described in Sections 2.3. and 2.4a.

5.2.3b **Medium** Two of the aliquots of medium that were removed from the incubation flask (see Section 2.6.) were assayed for $^{125}$I-PVP radioactivity as described in Section 2.4a. The remaining aliquot was diluted with distilled water so that the leupeptin concentration fell within the range of the standard curve, and assayed for active leupeptin.

5.2.4. Recovery of Leupeptin

The amount of leupeptin recovered from incubated yolk-sac tissue was estimated by adding a known amount of leupeptin to intact, unincubated tissue then preparing the tissue for assay as described in Section 5.2.3a. The leupeptin content of the tissue was determined using the Z-Phe-Arg-AMC-based assay and the observed value expressed as a percentage of that added. Recovery of leupeptin from a solution of 10% calf serum (heat inactivated) was also measured. In some recovery experiments a solution of a mixture of peptides (Gly-Tyr and Tyr-Arg, each at a concentration of 1mg/ml) was added to the yolk sac instead of Triton x-100 (0.1%), before addition of leupeptin and homogenization, in order to try to displace leupeptin from any non-specific peptide-binding sites in the homogenate.

5.2.5. Loss of Leupeptin from Pre-Loaded Yolk-Sac Tissue

Yolk sacs were incubated for 3h with leupeptin (100µg/ml) and $^{125}$I-PVP (2-4µg/ml) to load the tissue with these substrates. All tissue was then removed from the flasks and rinsed in three changes of fresh, gassed, warm medium (approximately 10ml each). Samples (3 x 1ml) of the loading medium were reserved for assay of leupeptin and radioactivity content, as described in Section 5.2.3b. The rinsed yolk sacs were transfered to flasks containing fresh, gassed, warm medium (7ml) (keeping together throughout the procedure the sets of three yolk sacs that were incubated in the same flask during the

loading phase of the experiment) and re-incubated for up to 5h. After re-incubation for a set period, the yolk sacs (three per flask) were removed and rinsed in cold 1% saline, then stored together at -20°C prior to preparation for assay of leupeptin and $^{125}$I-PVP content, as described in Section 5.2.3a. One set of three yolk sacs was not re-incubated, but instead was used (after rinsing) to determine the initial leupeptin content of the tissue after loading. In some experiments samples of re-incubation medium (3 x 1ml) were also reserved for assay of $^{125}$I-PVP radioactivity. The amount of active leupeptin remaining within the yolk sac after re-incubation was calculated in terms of µl of medium whose contained leupeptin had been captured per mg protein and compared with that present in yolk sacs that had not been re-incubated. The difference betwen the two was expressed as a percentage of leupeptin lost:-

$$\%\text{Leupeptin lost} = \frac{\text{Amount present (µl/mg) after loading} - \text{Amount present (µl/mg) after re-incubation}}{\text{Amount present (µl/mg) after loading}}$$

The amount of $^{125}$I-PVP released into the re-incubation medium was also expressed in terms of µl/mg, and checked for correlation with the amount lost from the tissue.

The effect of an inhibitor of lysosomal proteolysis, ammonium chloride (10mM), on the loss of leupeptin and $^{125}$I-PVP from yolk sacs was determined. The experimental procedure was as described above except that ammonium chloride was added to the re-incubation medium at a final concentration of 10mM.

5.2.6. Loss of $^{125}$I-BSA$_{fd}$ from Yolk-Sac Tissue

In the experiment described in Section 5.2.6., loss of leupeptin was compared with the loss of a non-degradable substrate, $^{125}$I-PVP, which can only be lost from the tissue by exocytosis and/or cell death. It was considered necessary to compare the loss of leupeptin with that of a degradable substrate, $^{125}$I-BSA$_{fd}$. Tissue was loaded with $^{125}$I-BSA$_{fd}$ by a method similar to that used in Section 5.2.6. Yolk sacs were incubated (2 per flask) in medium 199(6.0ml) and $^{125}$I-BSA$_{fd}$ stock solution(1.0ml) for 3h then rinsed in three changes of warm medium and re-incubated in flasks containing fresh, warm, gassed medium 199 (7ml). At intervals up to 5h, duplicate samples were taken by removing the 2 yolk sacs from a flask, rinsing them in three changes of cold 1% saline and digesting them separately in NaOH (1M, 5ml). Aliquots of the digest were analysed for radioactivity and for protein content. The effect of ammonium chloride (10mM) on the loss of $^{125}$I-BSA$_{fd}$ from yolk-sac tissue was determined by including ammonium chloride in the re-incubation medium.

## 5.3. RESULTS

### 5.3.1. Standard Curve for Z-Phe-Arg-7-AMC-Based Leupeptin Assay

The standard curve for this assay covered a range of leupeptin concentrations between 0.1 and 50µg/ml (ie 0.001 to 0.5µg leupeptin per assay). This corresponded to approximately 10% to 99% inhibition. The concentration of leupeptin in the yolk-sac samples was usually between 0.1-2.0µg/ml, any samples found to be above this range were diluted with distilled water to approximately 1.0µg/ml and re-assayed. (Incubation medium was routinely diluted 100-fold before assay.) This allowed the middle portion of the standard curve to be used, corresponding to 10 to 70% inhibition. A plot of 'percentage inhibition' against 'concentration of leupeptin in the test solution added to the assay (µg/ml) is shown in Fig. 5.1. The graph is non-linear, suggesting that association between leupeptin and trypsin was non-stoichiometric at these low concentrations (Bergmeyer, 1984, see also Chapter 4).

The standard curve was transformed into a linear form by plotting the probit of the percentage residual activity against the natural log of the concentration of leupeptin in the test solution. This plot is shown in Fig.5.2. The degree of scatter about the mean value of probit percentage residual activity for each concentration of leupeptin was generally greatest at the higher and lower concentrations. [Probit values give the best linear transformation when the percentage values are close to 50% (Colquhoun, 1971), hence the very high and low percentage residual activity given by the extreme concentrations of leupeptin would not necessarily be expected to fall exactly on the line of best fit.]

An attempt was made to fit a curve to the non-probit-transformed data using curve-fitting programs in a package written for the GEC 4080 computer at Keele, and a curve-fitting program written for use on the

1906A/7600 computer at Manchester. The standard curve data were plotted in terms of percentage residual activity against either leupeptin concentration (µg/ml) or natural log of the leupeptin concentration. The computer-fitted curves that most closely fitted the data are shown in Figs. 5.3, and 5.4. A major disadvantage of the computer-fitted curves was the difficulty in converting observed percentage residual activities to the corresponding values of leupeptin concentration. No convenient means of determining values from such curves was available (other than estimation by eye, which would be both inaccurate and time-consuming). Since the 'fit' of the computer-fitted curves did not differ greatly from that of the probit-transformed straight-line plot, it was decided to use the linearized standard curve to calculate the leupeptin content of the experimentally derived samples.

For reasons that are not understood, the uninhibited rate of reaction tended to increase over each experimental session. Measured in terms of centimeters deflection across the chart recorder per minute, the rate would increase by up to 10% over the entire session (lasting up to 8 hours). It was therefore necessary to monitor the uninhibited rate several times throughout the session, and use a mean value of those uninhibited rates determined at approximately the same time as the assays containing leupeptin, when calculating the percentage residual activity.

Although the uninhibited reaction proceeded linearly over the period of monitoring, the assays containing leupeptin tended to give a non-linear trace. The rate of reaction slowed slightly with increasing time. The decrease in reaction rate over the period of monitoring may have been partly attributable to a decrease in temperature during the reaction. [The assay mixtures in the cuvettes were equilibrated together in a water-bath at 25°C, and the substrate

and buffer solutions were stored at this temperature also. However, the fluorimeter was not thermostatted, therefore the temperature of the assay mixture may have dropped over the period of monitoring, particularly since small assay volumes were used. The ambient temperature of the room in which the fluorimeter was housed differed over the entire series of experiments, from about 15°C to 22°C.]

5.3.2. Recovery of Leupeptin

The chosen method of preparation of yolk-sac tissue for use in these experiments gave better recovery of leupeptin than other methods tested. However, the recoveries obtained using the homogenization and TCA precipitation method as described in Section 5.2.3a, were not ideal. Unlike the corresponding results in the BAPNA-based method, full recovery was never achieved and the amounts recovered (expressed as a percentage of the amount added) were very variable. A plot of percentage recovery against amount of leupeptin added to the tissue is shown in Fig.5.5. The amount recovered when 5µg of leupeptin was added varied between 1.3µg to 3.4µg, corresponding to 26 to 68% recovery.

The percentage recovered did not seem to depend either on the amount of leupeptin added to the tissue (mean percentage recovery on addition of 5µg leupeptin was 47.6% (n=12) whereas on addition of 20µg and 30µg leupeptin, recovery was 56.8% and 38.8%, respectively), or on the concentration of homogenate protein present before TCA precipitation. When leupeptin was added to a 10% solution of heat-inactivated calf-serum the percentage recovery was greatly increased, possibly as a result of a glycoprotein trypsin inhibitor present in the serum, which may be incompletely precipitated by TCA. It was hoped that addition of an excess of other small peptides might increase the recovery of leupeptin by displacing leupeptin from any

non-specific binding sites on the homogenate macromolecules before precipitation. However, the presence of Gly-Tyr and Tyr-Arg had no effect on the observed recoveries. Manipulation of the tissue and homogenate (eg transfer of small volumes of homogenate from homogenizing vessel to the vial to which the TCA was added and measurement of small volumes of homogenate and TCA-solubles) producing incomplete recovery of the total volumes involved may have contributed to the poor recovery.

The results were too scattered to construct a calibration curve from which the amount of leupeptin likely to be present within yolk sacs incubated with leupeptin could be determined from the amount detected in such yolk sacs (c.f. Chapter 4 results). It was therefore decided to use the overall mean value of the percentage recovery to give a correction factor. The percentage recovery did not vary greatly with the amount of leupeptin added, so the correction factor should be applicable to the entire range of amounts of leupeptin detected. The mean percentage recovery was 48.7% (n = 30, standard deviation = 3.0) hence the correction factor used to adjust the leupeptin content of all yolk sacs in subsequent experiments was 2.05.

Addition of 10µl of TCA (2.5% w/v solution) or medium (diluted 100-fold) to the assay did not affect the rate of the reaction.

### 5.3.3. Uptake of Leupeptin and $^{125}$I-PVP

Leupeptin accumulation was monitored in yolk sacs that were incubated in medium containing leupeptin at concentration of 100 and 200µg/ml. In experiments performed at a leupeptin concentration of 100µg/ml, $^{125}$I-PVP was also present in the medium, and uptake of $^{125}$I-PVP was monitored simultaneously in these yolk sacs to determine the rate of fluid-phase pinocytosis. Uptake of $^{125}$I-PVP and

accumulation of leupeptin (at an extracellular concentration of 100µg/ml) is shown in Fig. 5.6 and accumulation of leupeptin at 200µg/ml is shown in Fig. 5.7. Results (corrected for recovery) are expressed as the mean ± S.D.

**5.3.3a. Uptake of $^{125}$I-PVP** Since the experimental design involved incubating the yolk sacs with both leupeptin and $^{125}$I-PVP it was necessary to check whether the presence of leupeptin had any effect on the rate of uptake of $^{125}$I-PVP. [The presence of $^{125}$I-PVP does not affect the rate of uptake of other molecules taken up by pinocytosis (Roberts et al., 1974), and is unlikely to affect any other membrane transport process since it does not bind to the cell membrane and is non-toxic. It was therefore considered unlikely that uptake of leupeptin would be affected by the presence of $^{125}$I-PVP.] Little difference was observed between the rate of uptake of $^{125}$I-PVP measured in the presence and absence of leupeptin (100µg/ml). The Endocytic Index in matched experiments was 3.51µl/h/mg yolk-sac protein in the absence of leupeptin and 3.44µl/h/mg in the presence of leupeptin.

**5.3.3b Accumulation of Leupeptin** After the tissue had been incubated with leupeptin (100µg/ml) for 1h the observed concentration of leupeptin in the TCA-soluble fraction of the homogenized yolk sac (uncorrected for recovery) was about 1 µg/ml, which was within the detection limit. The results obtained using leupeptin at a concentration of 200µg/ml were very scattered hence results can only be interpreted partially. Results obtained using leupeptin at a concentration of 100µg/ml were considerably less scattered, the standard deviation ranging from 0.86 to 2.23 at each point.

At both concentrations, the amount of leupeptin within the tissue reached a steady-state, when no further accumulation of the active inhibitor occurred. This steady-state was reached after about

3h with leupeptin at 100µg/ml. The tissue was still pinocytically, and hence metabolically, active after these times (as shown by the continued linear uptake of $^{125}$I-PVP up to 8h) indicating that the lack of accumulation was unlikely to be caused by the cessation of pinocytosis or active transport. The most likely explanation is that uptake of leupeptin continued throughout the incubation period, but, as time progressed, the leupeptin was lost from the tissue at the same rate as its entry. (Further experiments were carried out to investigate loss of leupeptin from yolk sacs loaded with leupeptin.) The steady-state concentration (corrected for recovery) of active leupeptin within the tissue was about 1.25 and 2.0µg/mg yolk-sac protein for leupeptin at concentrations of 100 and 200µg/ml medium, respectively.

The initial rate of accumulation of leupeptin at 100µg/ml (over the first 3h) can be compared with that of the fluid-phase pinocytic marker $^{125}$I-PVP. These rates were very similar, suggesting that leupeptin may possibly enter the tissue by pinocytosis, like $^{125}$I-PVP.

The effect of an inhibitor of pinocytosis on accumulation of leupeptin was determined. A plot of uptake of leupeptin (100µg/ml) and $^{125}$I-PVP in the presence of ammonium chloride (20mM) is shown in Fig 5.8. Uptake of $^{125}$I-PVP was strongly inhibited, indicating that pinocytosis had been abolished by the ammonium choride. No accumulation of leupeptin within the tissue occurred, suggesting that uptake of leupeptin was also inhibited. The degree of inhibition was similar to that observed for $^{125}$I-PVP. Since ammonium chloride is thought to affect tissue-processes other than pinocytosis, an attempt was made to monitor accumulation of leupeptin using an alternative inhibitor, low temperature. No leupeptin was detected in the tissue (by this method of assay) after incubation for up to 8h at this

temperature. Uptake of $^{125}$I-PVP was also very strongly inhibited. (Tissue levels were only just detectable by γ-counting; results not shown).

5.3.4. Loss of Active Leupeptin from Pre-Loaded Yolk-Sac Tissue

Tissue was loaded by incubating with leupeptin (100μg/ml) and $^{125}$I-PVP for 3h, (thus achieving the maximum, steady-state concentration of leupeptin within the tissue) then re-incubated in substrate-free medium for up to 5h. The amounts of leupeptin and $^{125}$I-PVP lost from yolk sacs, expressed as a percentage of that present in yolk sacs immediately after loading, are shown in Fig. 5.9. [The percentage of $^{125}$I-PVP lost from yolk sacs correlated well with the percentage detected in the medium, therefore only loss from the tissue will be discussed here.] For most experiments, the loss of $^{125}$I-PVP was only monitored in yolk sacs that had been loaded with both leupeptin and $^{125}$I-PVP (ie loss of both substrates was monitored simultaneously) but in one experiment loss of $^{125}$I-PVP was also monitored in separate yolk sacs that had not ben exposed to leupeptin, to check whether the presence of leupeptin within the tissue affected the rate of loss of $^{125}$I-PVP. No difference was observed between loss of $^{125}$I-PVP in the presence and absence of leupeptin.

Very little $^{125}$I-PVP was lost from the tissue during reincubation (approximately 5%). Since $^{125}$I-PVP can only be released from the tissue by exocytosis and/or cell lysis (Williams *et al.*, 1975a) the results indicate that these processes did not occur to any great extent during the 5h re-incubation period. In contrast, 90% of the active leupeptin was lost from the tissue after about 3h reincubation, hence 85% of the loss must have been caused either by inactivation of leupeptin within the tissue or by release of intact

leupeptin by a mechanism other than exocytosis/cell lysis.

The steady-state concentration of leupeptin after the 3h loading incubation period was about 1.25µg/mg yolk-sac protein (see Section 5.3.3.). Hence, assuming a total of about 12mg protein per yolk-sac sample (which is a typical mass for a set of three yolk sacs), the 90% loss of leupeptin corresponds to 13.5µg leupeptin. An approximate rate of loss of leupeptin over the first 3h of re-incubation can thus be calculated as 0.375µg leupeptin/mg yolk sac protein/h.

If the loss of leupeptin activity from the tissue was caused only by release of intact leupeptin from the tissue, the final concentration of active leupeptin in the re-incubation medium would have been about 2µg/ml, which is well within the detection limits of the assay. However, when an attempt was made to assay the re-incubation medium, little or no active leupeptin was detected.

### 5.3.5. Effect of Ammonium Chloride (10mM) on Loss of $^{125}$I-BSA$_{fd}$, $^{125}$I-PVP and Active Leupeptin from Pre-loaded Yolk-Sacs

Ammonium chloride (10mM) was included in the re-incubation medium to inhibit lysosomal proteolysis, and hence inhibit the hydrolysis of degradable substrates within the lysosomes of the pre-loaded yolk sacs. This would inhibit loss of such a substrate from the tissue.

The results obtained when the tissue was loaded with $^{125}$I-BSA$_{fd}$ and re-incubated in the presence and absence of ammonium chloride (10mM) are shown in Fig. 5.10; these are typical of results for proteins. In the absence of ammonium chloride, $^{125}$I-BSA$_{fd}$ is rapidly lost from the tissue over the first hour of re-incubation. Only about 65% of the $^{125}$I-BSA$_{fd}$ was lost, no further loss occurred over the remainder of the 5h re-incubation period. (Similar results were

also observed by Livesey (1978) and may possibly be caused by $^{125}$I-BSA$_{fd}$ remaining associated with the yolk sac in the intercellular spaces, where it is not degraded.) When ammonium chloride was present in the re-incubation medium both the rate and amount of $^{125}$I-BSA$_{fd}$ loss were lowered. A maximum of about 45% was lost from the tissue, and the steady-state was only reached after a 2h re-incubation period. These results indicate that the presence of 10mM ammonium chloride in the re-incubation medium was sufficient to inhibit hydrolysis of $^{125}$I-BSA$_{fd}$; the undegraded protein remained trapped within the yolk-sac lysosomes.

The effects of ammonium chloride on the loss of both leupeptin and $^{125}$I-PVP (measured simultaneously) are shown in Fig. 5.11. Ammonium chloride had no effect on the amount of $^{125}$I-PVP lost from the tissue, indicating that exocytosis and cell death were unaffected by the inhibitor. (It also suggests that the permeability of the cell membranes towards this large substrate, mean mol. mass = 40,000, was unchanged.) The observed effect of ammonium chloride on the loss of leupeptin from the yolk sacs was unexpected. Loss of leupeptin was accelerated, virtually all leupeptin was lost from the tissue within one hour. The rate of loss was calculated to be about 1.25µg leupeptin/mg yolk sac protein/h. This increase in the rate of loss could not be attributed to increased exocytosis or cell death, and it occurred even though proteolysis was inhibited under the same conditions.

## 5.4. DISCUSSION

### 5.4.1. Trypsin-Mediated Hydrolysis of Z-Phe-Arg-7-AMC as a Method for Assay of Leupeptin

In this assay the detection limit for leupeptin was lowered by decreasing the amount of trypsin in the reaction mixture, so that less leupeptin was required to achieve an observable inhibition. The readily-hydrolysed substrate and the highly fluorescent reaction product allowed the reaction to be monitored using this lower enzyme concentration. The major advantage of the assay was that it enabled the very small amounts of leupeptin present within the yolk-sac tissue to be detected quantitatively. Accumulation of leupeptin could therefore be monitored and compared with the uptake of a marker molecule.

However, a number of problems were encountered when using this assay method. (Some of the practical problems and means of overcoming them were discussed in the results section). An important drawback was that of incomplete recovery of leupeptin from yolk-sac tissue. Several different methods of preparing tissue for assay were tested in an attempt to improve the recovery (see Appendix 2). However, even using the method that gave the best recovery (ie TCA precipitation), only about 50% of leupeptin added to the tissue in control experiments was detected, and this value was variable. The reason for the poor recovery was not identified. Possibly leupeptin became bound to proteins in the yolk-sac homogenate and was co-precipitated with them on addition of TCA. Since addition of dipeptides did not improve recovery, any such putative binding sites must be fairly specific for leupeptin. (Experiments conducted to test whether leupeptin became appreciably bound to a yolk-sac homogenate are discussed in Chapter 6).

Another potential problem associated with the method was that the $K_m$ for the association of trypsin with Z-Phe-Arg-7-AMC was not known, hence it was not possible to predict whether competition between the substrate and leupeptin would occur. (Ideally the $K_i$ for the interaction between an inhibitor and enzyme should be $10^3$- to $10^4$-fold smaller than the $K_m$ for the substrate-enzyme interaction, see Section 4.4.1). The standard curve was non-linear, suggesting that the interaction between leupeptin and trypsin was non-stoichiometric, as would be expected if competition did occur. However, the inhibition observed in samples of unknown leupeptin concentration were compared with those observed in a standard curve for which the concentrations of leupeptin were known. Any competition between substrate and inhibitor would occur in both standard curve and experimental sample, and would thus not affect the conversion of the observed percentage inhibition to a quantity (ng) of leupeptin.

### 5.4.2. Discussion of Experimental Results Obtained Using the Z-Phe-Arg-7-AMC Based Leupeptin Assay

Only intact leupeptin can be detected by this enzyme assay, therefore the observed rate of entry of leupeptin corresponds to the rate of accumulation of intact, active leupeptin, rather than the total rate of uptake.

The results in Fig.5.6 show that accumulation of leupeptin occurred at approximately the same rate as fluid-phase pinocytosis, suggesting that this is probably the main route of entry. However, as it was possible that the net rate of accumulation observed was less than the true rate of uptake, additional routes of entry could not be entirely ruled out.

Further evidence for fluid-phase pinocytosis being an important

mode of entry was given by the results from experiments in which pinocytosis was inhibited by ammonium chloride or low temperature. In such experiments, tissue accumulation of leupeptin was inhibited to the same extend as fluid-phase pinocytosis. However, the mechanism of action of ammonium chloride as an inhibitor of pinocytosis is not established and several additional effects in yolk sacs are well documented (Livesey et al., 1980). It is therefore possible that the inhibition of leupeptin accumulation in the presence of ammonium chloride may not have been caused entirely by an inhibition of pinocytosis. Likewise, the total inhibition of accumulation observed for tissue incubated at 5°C could have been attributed both to inhibition of pinocytosis and/or to other membrane immobilizing effects. Nevertheless, ammonium chloride and low temperature would not be expected to totally inhibit any passive diffusion across the cell membrane, therefore the strong inhibition of accumulation of leupeptin afforded by these agents suggests that passive diffusion is not a major route of entry for leupeptin into yolk-sac tissue.

After 3h incubation with leupeptin (100µg/ml) (and about the same period with leupeptin at 200µg/ml) the amount of active leupeptin in the tissue reached a constant value of approximately 12 or 10µl/mg yolk-sac protein for leupeptin at an extracellular concentration of 100 and 200µg/ml respectively. This corresponded to about 1.25 and 2.0µg leupeptin per mg of tissue protein.

The generation of a steady-state in which the quantity of leupeptin associated with the tissue remains constant in spite of continuing pinocytic capture, requires loss of active leupeptin from the tissue. This could arise either by inactivation and/or degradation within the tissue or by release of intact leupeptin from the tissue. The rate of loss must equal the rate of entry. This would occur if the rate of inactivation was not rate-limiting or if

leupeptin left the tissue at the same rate as entry (as would be expected for non-concentrative passive or facilitated diffusion).

Loss of leupeptin activity from tissue that had been loaded with leupeptin (100µg ml) was monitored in an attempt to identify the mode of loss. The maximum rate of loss was calculated to be about 0.375µg/mg yolk-sac protein/h, which is equivalent to 3.75µl/mg yolk-sac protein/h. This is similar to the rate of accumulation of leupeptin observed over the first three hours during the uptake experiment (ie about 3.44 µl/mg/h).

Leupeptin could not be detected in the re-incubation medium, which suggested that some or all of the leupeptin must have been inactivated rather than released from the tissue. [Only 5% of loss of substrate can be attributed to exocytosis and/or cell death, as indicated by the $^{125}$I-PVP results. This corresponds to 0.75µg leupeptin, giving a concentration in the re-incubation medium of 0.1µg/ml, which would be only just detectable using this assay.]

The inclusion of ammonium chloride (10mM) in the re-incubation medium was shown to inhibit lysosomal proteolysis of $^{125}$I-BSA$_{fd}$, as less radioactivity was lost from the tissue in the form of the membrane-permeable degradation products of $^{125}$I-BSA$_{fd}$. Ammonium chloride did not appear to greatly affect the rate of release of $^{125}$I-PVP, suggesting that exocytosis, membrane permeability to towards large molecules, and cell lysis were not affected. However, in the presence of ammonium chloride the rate of loss of leupeptin from the tissue was increased three-fold and the extent of loss increased from 90% (observed in the absence of ammonium chloride) to 100%.

At least two possible explanations can be forwarded to explain the enhanced loss of leupeptin:

a) Leupeptin may have been inactivated and/or degraded more rapidly in the presence of ammonium chloride.

If leupeptin inactivation/degradation was entirely lysosomal, stimulation in the presence of a lysosomotropic agent would suggest that the enzyme(s) responsible must be maximally active near neutral pH. Several such enzymes that may be capable of cleaving the peptide bonds of leupeptin exist, eg lysosomal aminopeptidase, lysosomal carboxypeptidase B, dipeptidylpeptidase I, (Barrett, 1977).

Alternatively, ammonium chloride may act by increasing the permeability of the lysosomal membrane, allowing access of leupeptin to sites and enzymes not normally available. [Small changes in the lysosomal membrane permeability are feasible because ammonium chloride causes vacuolation of lysosomes, and the distended membrane may become more 'leaky' towards small molecules.] It is known that leupeptin normally has access to lysosomal sites (because it inhibits lysosomal proteolysis), therefore any change in membrane permeability is most likely to affect movement of leupeptin from lysosomes to the cytoplasm. Cytosolic peptidases could then inactivate/degrade the leupeptin. This possible explanation of the effect of ammonium chloride would only hold if leupeptin did not normally have access to the cytoplasm.

b) Leupeptin may have been released (intact) from the tissue more rapidly in the presence of ammonium chloride.

The rate of release of leupeptin may increase if the ammonium chloride increased plasma membrane permeability and/or stimulated membrane transport systems, and if the rate of exocytosis or diacytosis of vesicles containing leupeptin was enhanced. This possibility was further investigated in Chapter 6.

To summarize, the assay gave clear results with good reproducibility. It was possible to detect the total tissue content of active leupeptin. The experiments described in this Chapter suggest that:-

i) Accumulation of leupeptin occurs at a rate similar to that of fluid phase pinocytosis.

ii) Accumulation of leupeptin appeared to be inhibited by ammonium chloride (20mM) and low temperature.

iii) Leupeptin activity was lost from the tissue, so that a steady-state concentration was reached after about 3 hours.

iv) Loss of leupeptin activity (but not 125I-PVP or 125I-$BSA_{fd}$) was enhanced in the presence of ammonium chloride (10mM).

Since it was necessary to apply a mean correction factor to all amounts of leupeptin measured in the TCA-soluble fraction of yolk sacs (to correct for poor recovery of leupeptin) the precise quantity of leupeptin within the yolk sacs may not correspond exactly to that shown in the results. However, the general pattern was quite clear, and since the correction factor was a constant, it should not alter the slope of the graph of accumulation of leupeptin against time (ie the rate of accumulation), when accumulation is expressed as µl of medium whose substrate content has been captured.

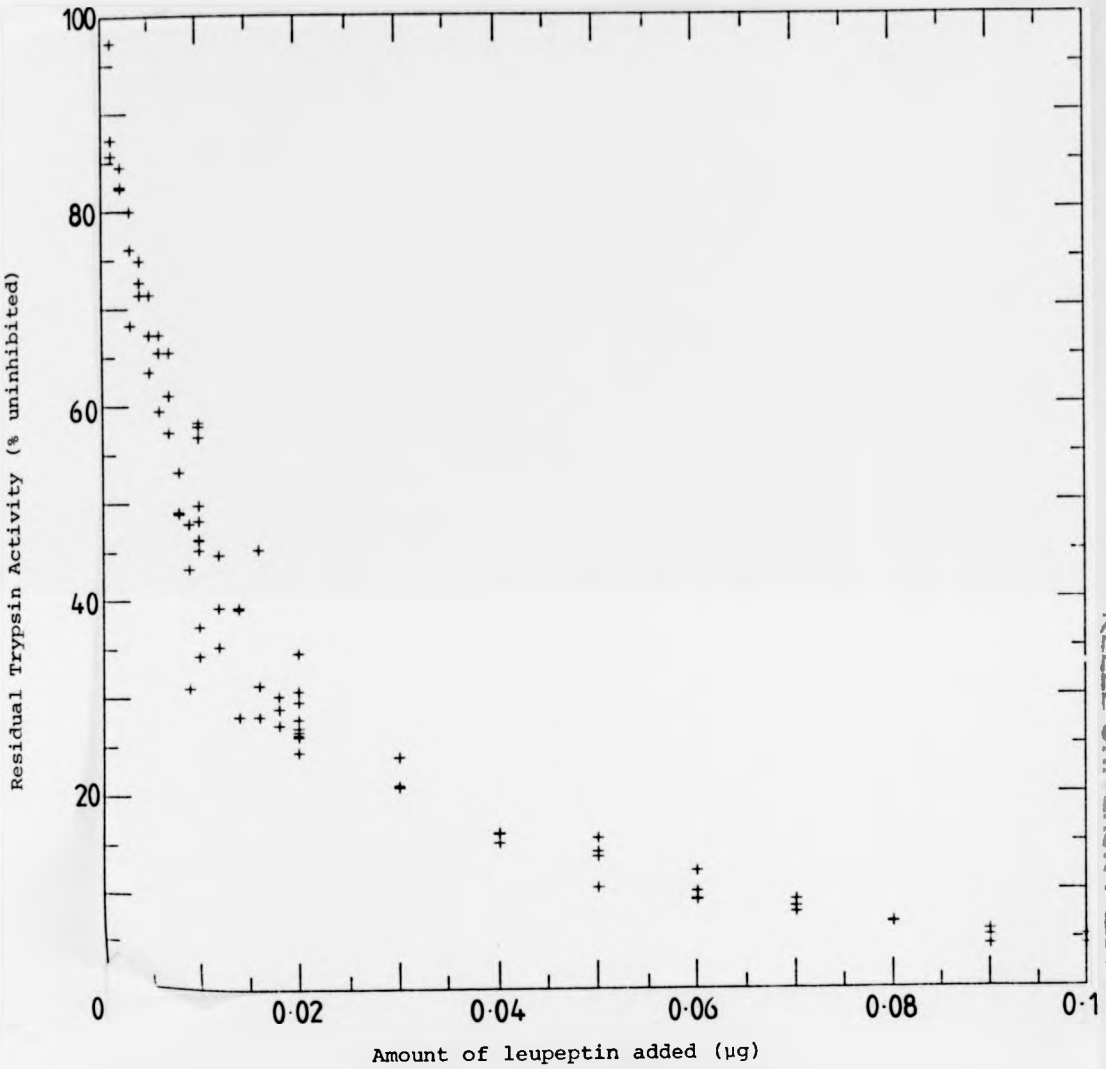





Fig. 5.1 Inhibition Curve for Trypsin and Leupeptin Using Z-Phe-Arg-AMC as the Substrate

The percentage of enzyme activity that remained when various amounts of leupeptin were added to a fixed amount of trypsin was determined by the enzyme assay described in Section 5.2.1. Each point represents a single determination.

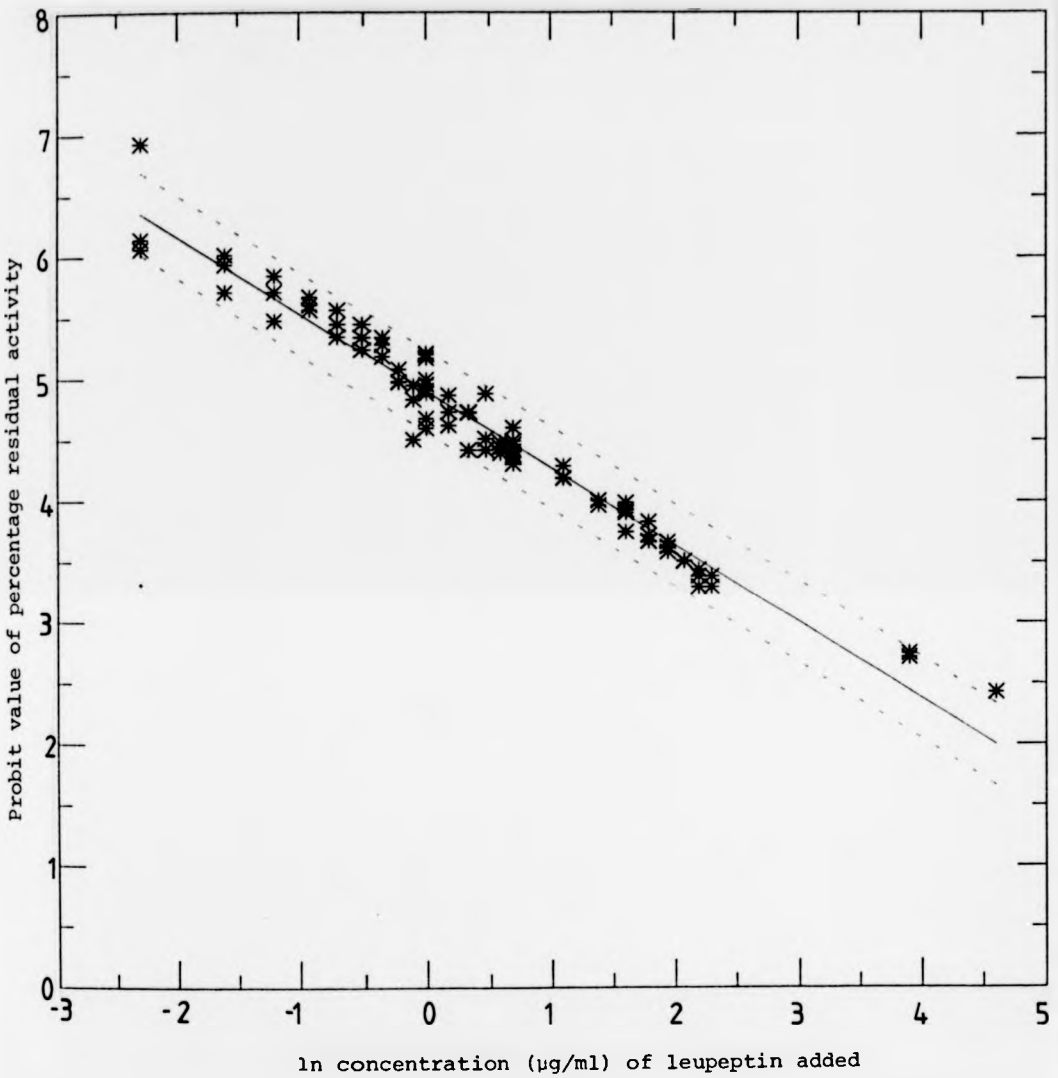



Fig. 5.2 Probit Transformation of the Inhibition Curve: Linearization to give Standard Curve

The probit value of the percentage residual activity of trypsin in the presence of various amounts of leupeptin (determined as described for Fig 5.1) was plotted against the natural logarithm of the concentration (µg/ml) of leupeptin added to the assay. Each point represents a single determination. The graph also shows a regression analysis giving the line of best fit and 95% confidence limits.

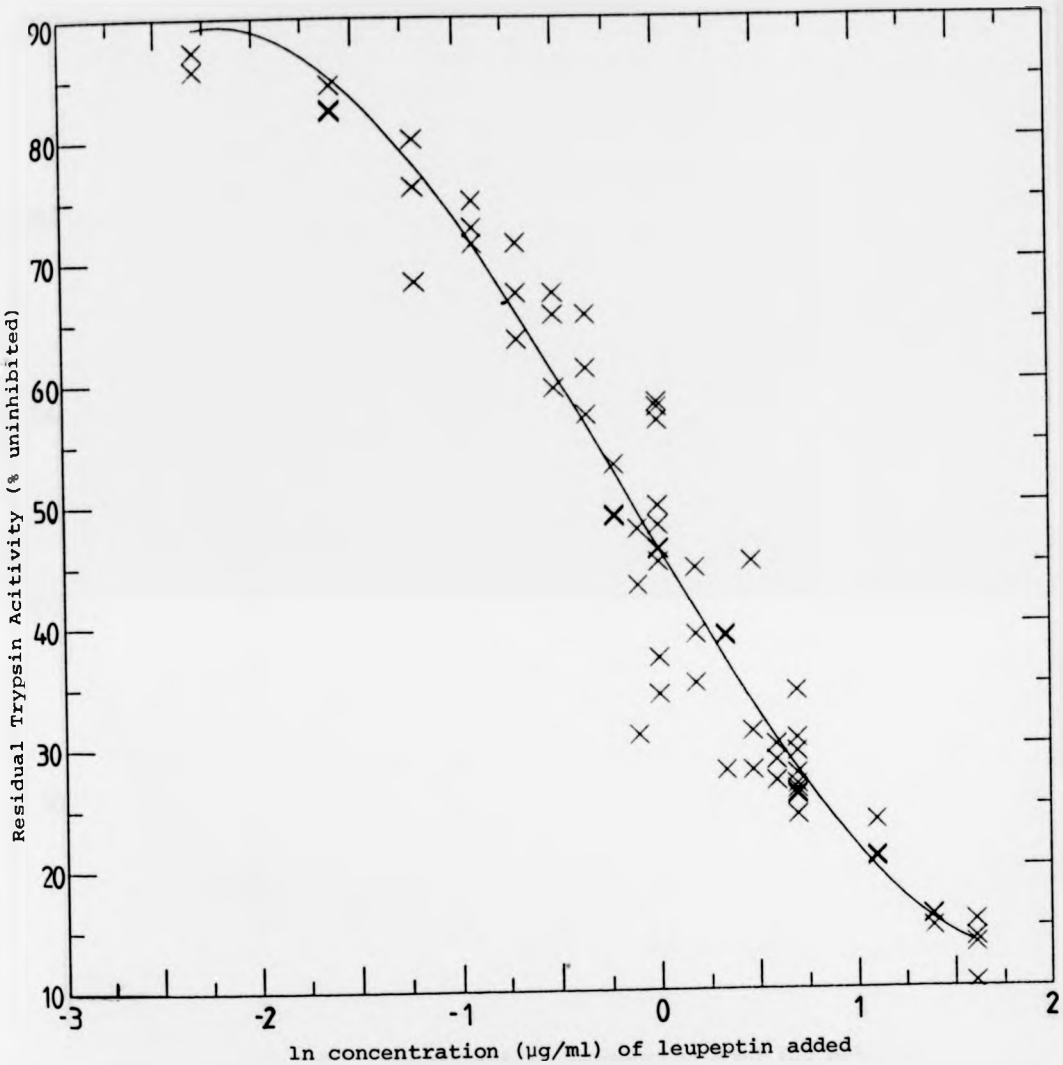





Fig. 5.3 Computer-Fitted Line of Best Fit Through Standard Curve Data: Percent Residual Activity against natural log. Leupeptin Concentration

The data given in Fig. 5.1 were plotted as percentage residual activity against the natural log of the concentration of leupeptin added, and the curve of best-fit calculated by computer.

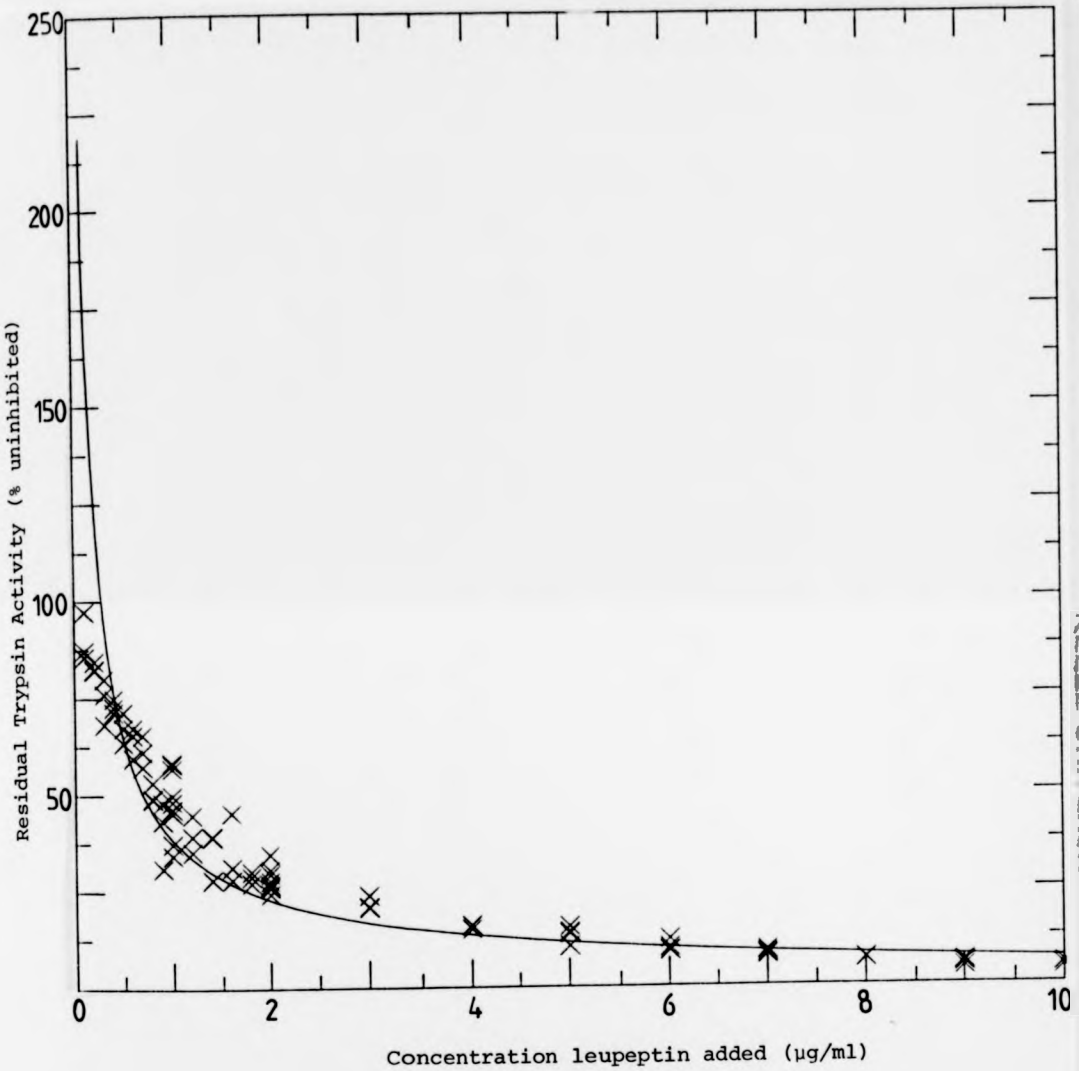
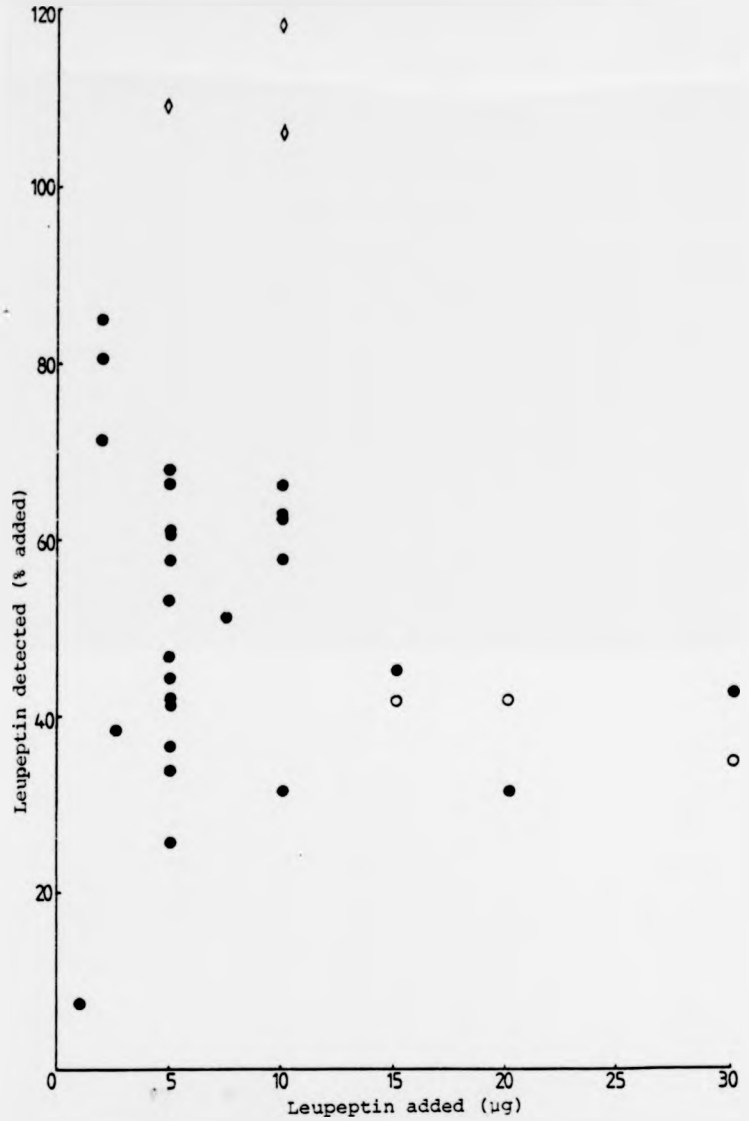





Fig. 5.4 Computer-Fitted Line of Best Fit Through Standard Curve Data: Percent Residual Activity against Leupeptin Concentration

A curve of best-fit was calculated by computer for the data given in Fig. 5.1.




● Recovery of leupeptin from yolk sac homogenate

○ Recovery of leupeptin homogenates in the presence of peptides

◊ Recovery of leupeptin from calf serum

Fig. 5.5 Recovery of Leupeptin from the TCA-soluble Fraction of a Yolk-Sac Homogenate

Yolk sacs were homogenized and known amounts of leupeptin added, as described in Section 5.2.4. The amount of leupeptin detected in the TCA-soluble fraction of the homogenate, measured as described in Section 5.2.1, was calculated as a percentage of that added. Peptides were added to some homogenates before TCA-precipitation. Recovery of leupeptin from calf serum, rather than yolk sac homogenate, was also measured. The plot shows results for individual determinations using different homogenates.

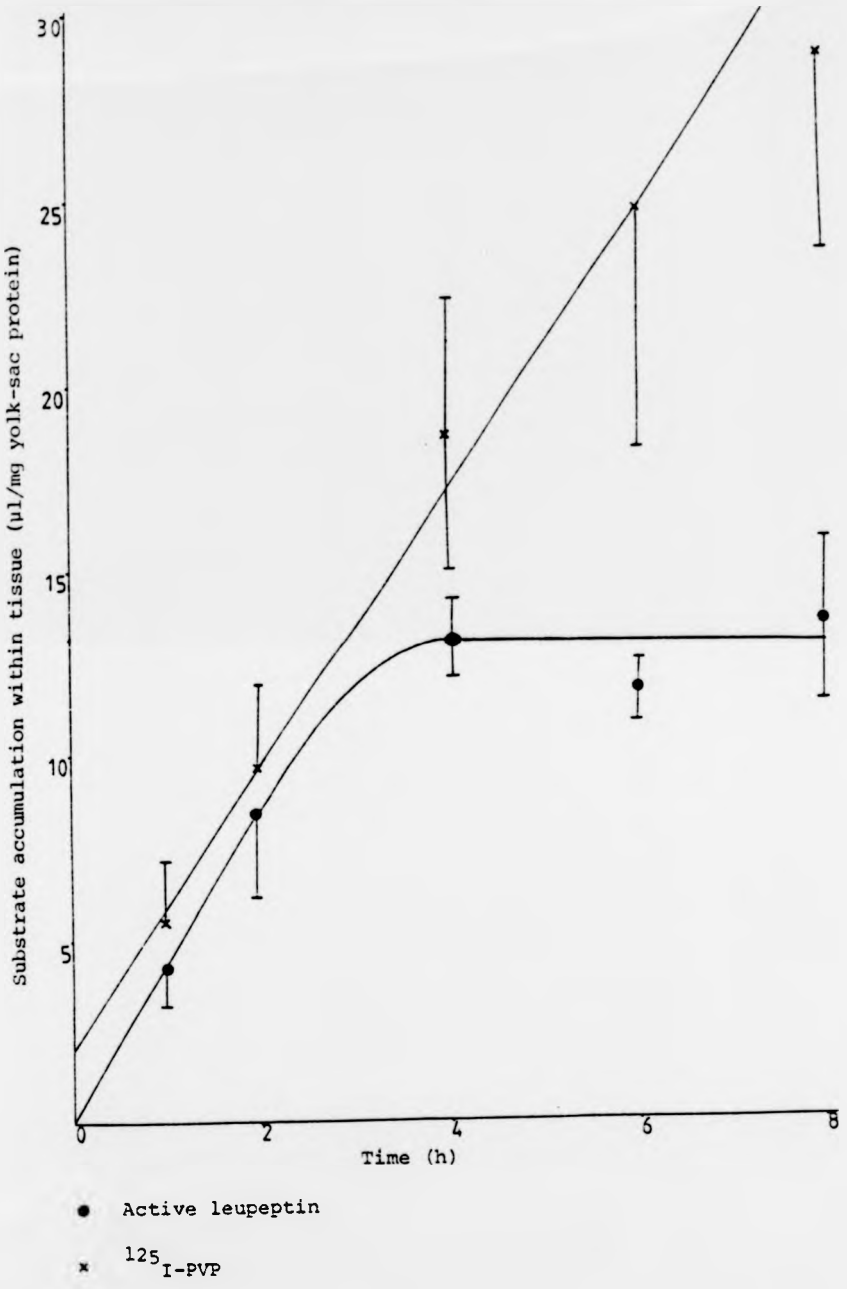



Fig. 5.6 Accumulation of $^{125}$I-PVP and Active Leupeptin Within Yolk Sacs

Yolk sacs were incubated with both leupeptin and $^{125}$I-PVP, as described in Section 2.6. Tissue and medium were assayed for leupeptin and radioactivity as described in Section 5.2.3. The amount of tissue-associated leupeptin was corrected for recovery, then substrate accumulation calculated as described in Sections 2.7a and 2.7d. The graph shows mean accumulation (± S.D.).

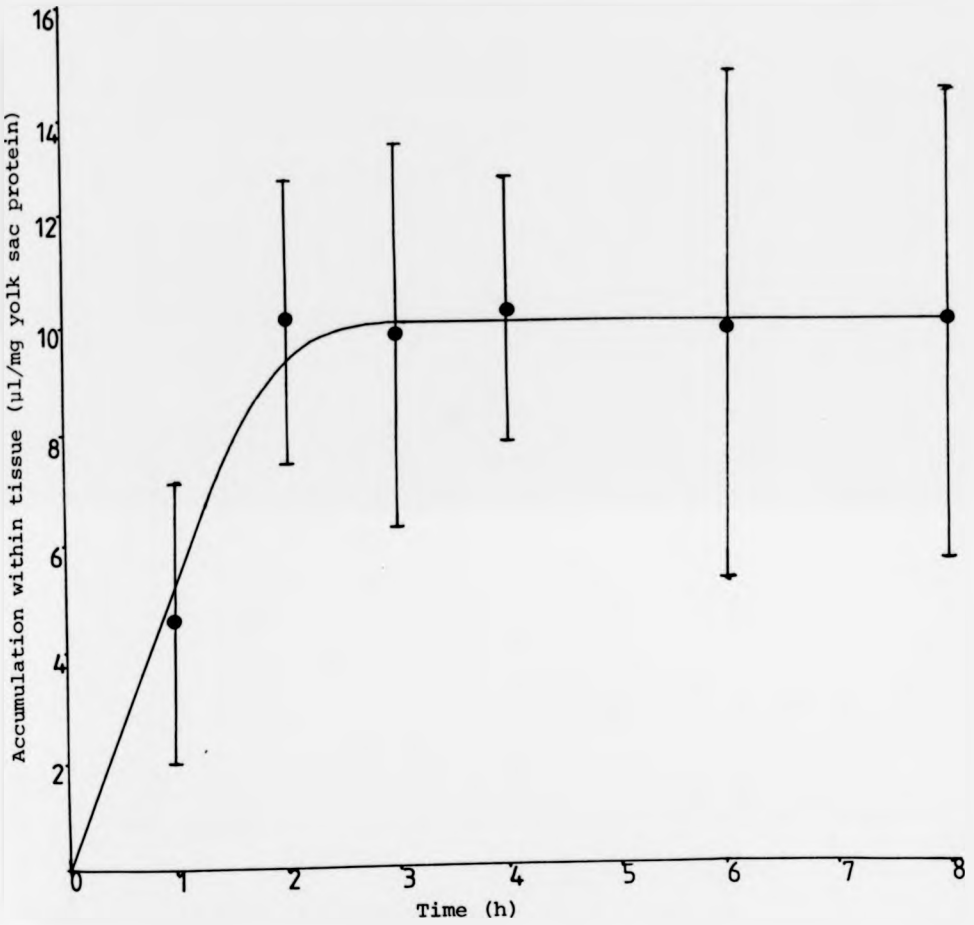



Fig. 5.7 Accumulation of Active Leupeptin Within Yolk Sacs with Leupeptin at a Medium concentration of 200 µg/ml

Yolk sacs were incubated with leupeptin (200 µg/ml) as described in Section 2.6, except that $^{125}$I-PVP was not present. Yolk sacs and medium were assayed for active leupeptin, and tissue associated leupeptin corrected for recovery. Accumulation of leupeptin within the tissue was calculated as described in Section 2.7d. The graph shows the mean results (± standard deviation).

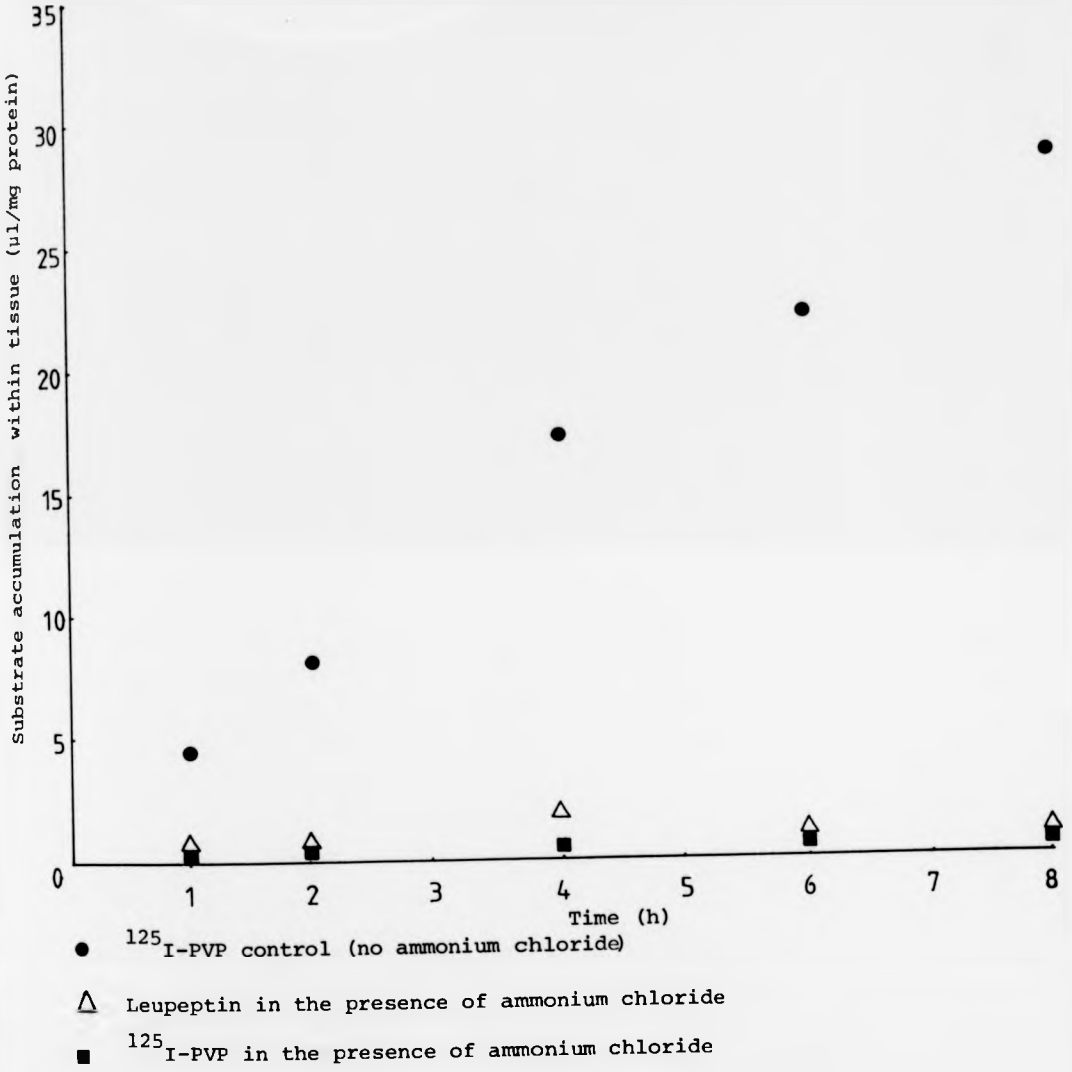



Fig. 5.8 Accumulation of $^{125}$I-PVP and Active Leupeptin Within Yolk Sacs Incubated in the Presence of Ammonium Chloride

Yolk sacs were incubated with both $^{125}$I-PVP (2-4 µg/ml) and leupeptin (100 µg/ml) in medium containing ammonium chloride (20 mM). $^{125}$I-PVP accumulation was also measured in the absence of ammonium chloride as a matched control. Tissue and medium were assayed for leupeptin and for radioactivity, then accumulation of substrates was calculated as described Fig. 5.6. The mean accumulation (± standard deviation for control) is shown.

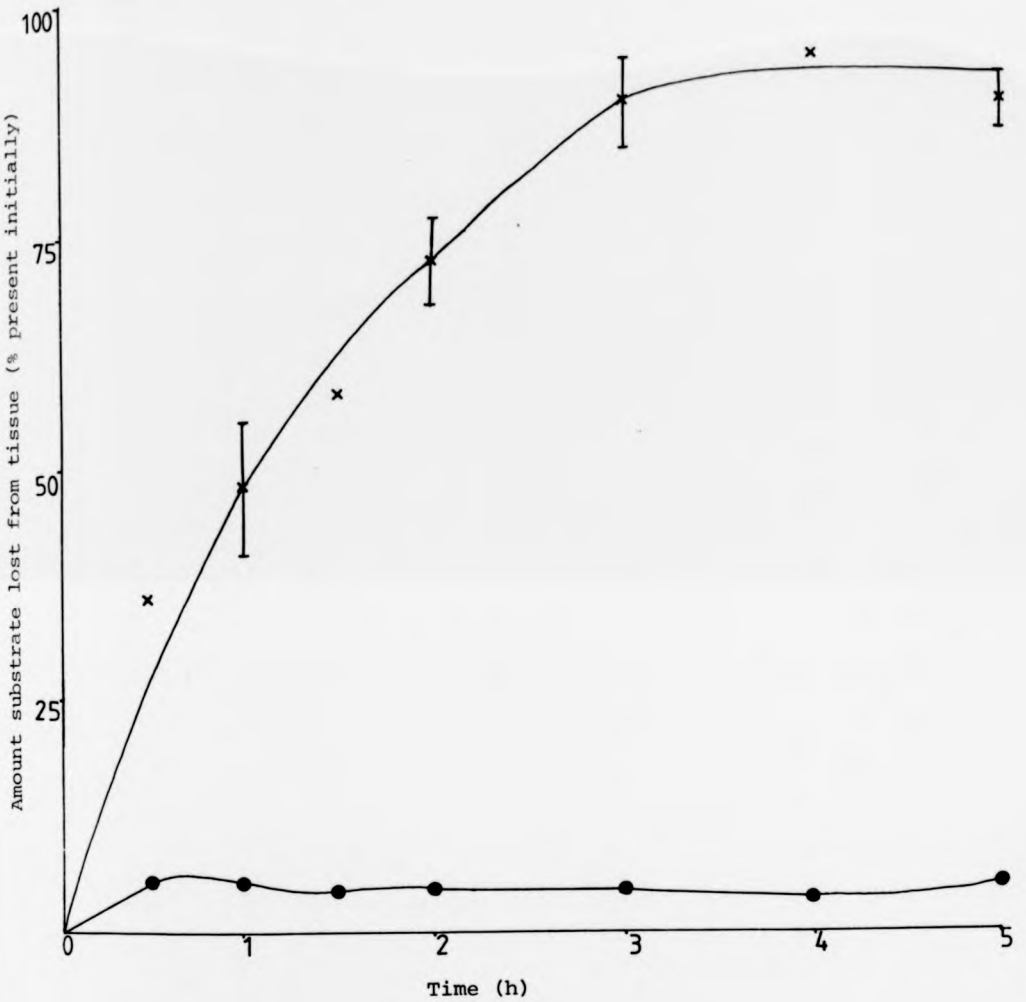
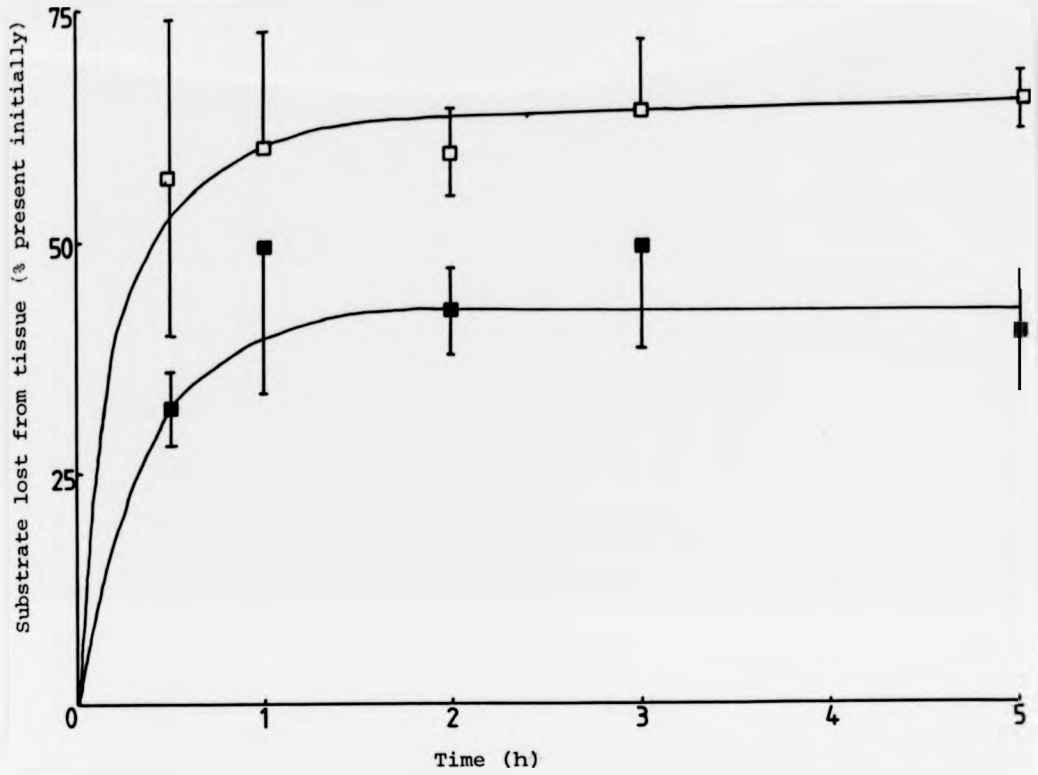



Fig. 5.9 Loss of $^{125}$I-PVP and Active Leupeptin from Pre-Loaded Yolk Sacs

Yolk sacs were incubated in the presence of $^{125}$I-PVP and leupeptin then re-incubated in substrate-free medium, as described in Section 5.2.5. The amount of substrate that remained in the tissue after incubation was calculated as described in Section 5.2.5. The graph shows the mean (± S.D., where apparent) amount of substrate lost, expressed as a percentage of that present at the start of re-incubation.




□ $^{125}$I-BSA in the absence of ammonium chloride

■ $^{125}$I-BSA in the presence of ammonium chloride

Fig. 5.10 Loss of $^{125}$I-BSA$_{fd}$ from Pre-Loaded Yolk Sacs in the Presence and Absence of Ammonium Chloride

Tissue was incubated with $^{125}$I-BSA$_{fd}$ then rinsed and re-incubated with and without ammonium chloride (10 mM). The amount of radioactivity that remained within the tissue after reincubation (calculated in terms of µl/mg protein) was expressed as a percentage of that present in tissue that had not been reincubated, as described in Section 5.2.6. The mean percentage ± standard deviation is shown in the diagram.

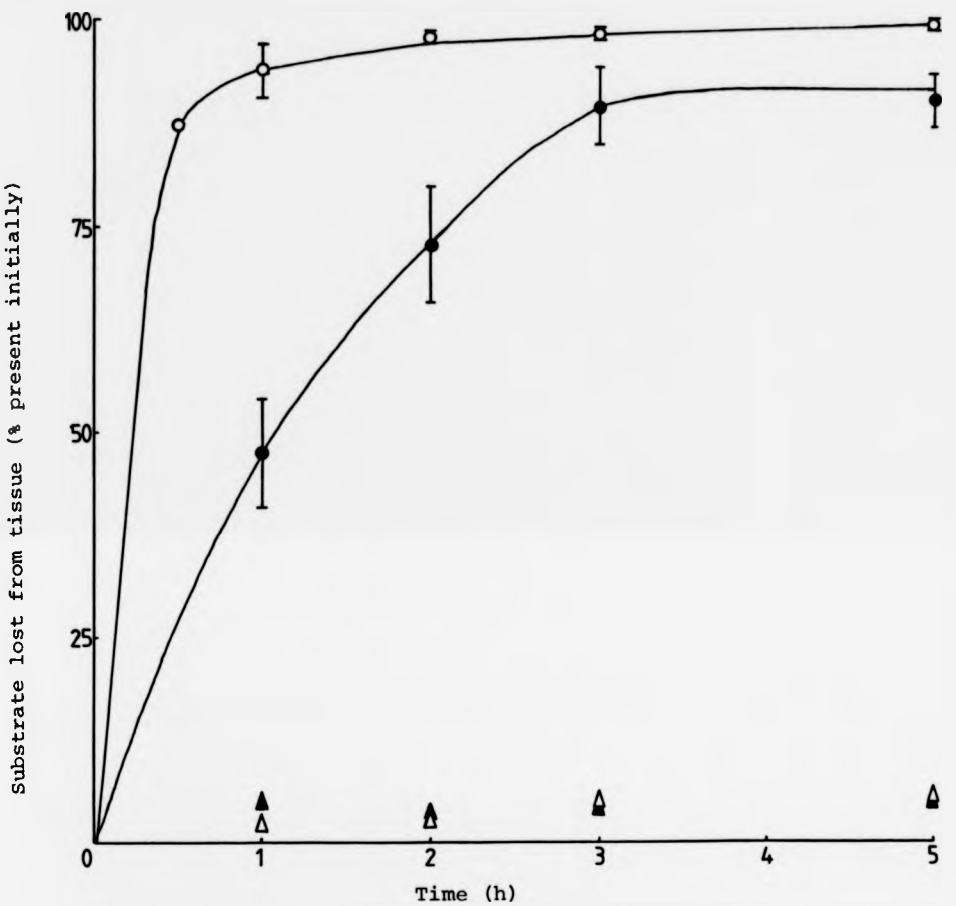



Fig. 5.11 Loss of $^{125}$I-PVP and Active Leupeptin from Pre-Loaded Yolk Sacs in the Presence and Absence of Ammonium Chloride

Tissue was treated as described in Fig. 5.9, except that ammonium chloride (10mM) was present during re-incubation. The percentage of substrate lost in the presence of ammonium chloride (mean ± S.D.) was plotted together with the data from Fig. 5.9 in order to illustrate the effect of ammonium chloride on the loss of active leupeptin and $^{125}$I-PVP from yolk sacs.

# CHAPTER 6

## RADIOMETRIC ($^{3}$H-MONITORING) METHOD OF LEUPEPTIN DETECTION

## 6.1. INTRODUCTION

The most frequently utilized method of quantitating uptake (or accumulation) is to use a substrate that has been radio-labelled. Uptake can then be monitored by counting the amount of radioactivity within the cells or tissue after incubation with tracer quantities of the radiolabelled substrate. Uptake is either expressed as c.p.m. per unit cell mass or number of cells (eg Ose et al., 1980; Besterman et al., 1981), or, by using the specific radioactivity of the substrate, c.p.m. can be converted to the amount of substrate captured (μmol or μg). Alternatively, uptake can be expressed as the volume of incubation medium (μl) whose contained substrate has been captured by the cells or tissue (eg Williams et al., 1975a; Bowers & Olszewski, 1972).

The radioisotopes most commonly used in biochemical investigations are $^{125}I$, $^{14}C$ and $^{3}H$. The first of these is a γ-emitter and hence is detected by using a crystal scintillation counter; $^{14}C$ and $^{3}H$ are β-emitters whose emissions are less penetrating hence they are detected by liquid scintillation counting. The isotopes $^{3}H$ and $^{14}C$ are usually incorporated into the normal molecular structure of a compound, so the labelled and non-labelled molecules are structurally indistinguishable. $^{125}I$ is generally covalently attached to molecules to form an iodinated derivative, hence the properties of the molecule may be affected.

In the work described in this chapter, the accumulation and release of leupeptin were investigated using a sample of tritiated leupeptin. [This substrate is not commercially available in a labelled form and the unlabelled molecule cannot be iodinated because the molecule does not contain tyrosine. The sample of $^{3}H$-leupeptin used was synthesized by Dr. J. Powers using a method of isotope-exchange (Bush et al., 1981), and was kindly donated by Dr.

R. Beynon, University of Liverpool.]

The many advantages and disadvantages inherent in using radiolabelled substrates will be considered in detail in the discussion section of this chapter. Briefly, the main advantage of using a radiolabelled substrate is the low detection limit that is possible if the substrate is available at high specific radioactivity. It was hoped that the lower detection limit for $^{3}$H-leupeptin would permit accumulation of leupeptin to be measured with greater sensitivity. If so, accumulation could be monitored over a wider concentration range, and small amounts of leupeptin associated with the tissue, either at early times during a time-course of accumulation or during inhibition studies, could be accurately quantified.

Many criteria have been used in the past to differentiate between the various modes of uptake of extracellular material into cells (ie passive diffusion, facilitated or active transport, fluid or adsorptive pinocytosis). However, similar criteria may be used to demonstrate different modes of uptake (see Chapter 1), therefore a combination of criteria must be used in order to decide on the mechanism of uptake. In this chapter, the effects of leupeptin concentration, temperature of incubation, and metabolic inhibitors on the rate of accumulation of $^{3}$H-leupeptin were all monitored in an attempt to distinguish between the various modes of uptake. Also the rate of accumulation of $^{3}$H-leupeptin was compared with the rate of uptake of a $^{3}$H-labelled marker for fluid-phase pinocytosis, $^{3}$H-labelled inulin (mol.wt.5,250).

$^{3}$H-Inulin has been used by Bowers & Olszewski (1972) to study fluid-phase pinocytosis in the Acanthamoeba. In the Acanthamoeba $^{3}$H-inulin obeyed all the requirements for a marker of fluid phase pinocytosis (discussed further in Section 6.4.3). It could not pass

through the cell membrane rapidly via diffusion or permeation; it was not metabolized; and it was taken up at the same minimal rate as other fluid pinocytosis markers, hence it does not appear to adsorb to the plasma membrane. Equivalent checks were made in the yolk-sac system before using $^{3}$H-inulin as a marker to compare the rate of accumulation of $^{3}$H-leupeptin with the rate of fluid-phase pinocytosis.

Release of radiolabelled material, from tissue or cells that have been preloaded with a radio-labelled substrate, can be monitored by measuring the appearance of the label in the re-incubation medium (Besterman et al., 1981; Dean & Jessup, 1982; Williams et al., 1975a). Ideally the nature of the released radiolabelled material should be known, so that the possibility of intracellular degradation of the substance can be assessed. (Unlike the enzyme assay based methods of detection, any inactivated $^{3}$H-leupeptin and/or $^{3}$H-labelled degradation products are detected.) Several attempts were made to find a convenient method for the separation of intact leupeptin from any degradation products (see Appendix 4).

The total amount of radioactivity released may give an indication of the mode of release. The time-course of release of $^{3}$H-leupeptin-derived radioactivity was compared with that of markers of pinocytosis that are released by exocytosis/cell death alone. If either leupeptin itself or leupeptin degradation products were released by a mechanism other than exocytosis/cell death, the kinetics of release would be expected to differ from those of these markers.

In results described in Section 5.3.6 it was observed that ammonium chloride enhanced the loss of leupeptin activity from yolk sacs. Several possible explanations were put forward: an increased rate of lysosomal inactivation or degradation; increased permeability

of the lysosomal membrane (leading to release of leupeptin from lysosomes followed by possible degradation by cytosolic peptidases); increased permeability or stimulation of transport through the plasma membrane (leading to release of leupeptin from the tissue). In the experiments reported in this chapter, the effect of ammonium chloride on the release of label derived from $^{3}$H-leupeptin (ie intact and/or degraded material) and of two non-degradable radiolabelled markers of fluid-phase pinocytosis was determined, to try to distinguish between these possibilities. The markers used were of different molecular sizes. It was thus hoped to detect any small changes in membrane permeability, as well as any changes in the extent of exocytosis/cell death, that may occur in the presence of ammonium chloride.

It was also noted in Chapter 5 that when leupeptin was added to a yolk-sac homogenate and the homogenate proteins precipitated with TCA, recovery of the leupeptin from the soluble fraction was poor. Possible explanations of this were discussed; one of these was that leupeptin bound to some of the homogenate proteins and was co-precipitated. This possibility was investigated using $^{3}$H-leupeptin. A mixture of $^{3}$H-leupeptin and homogenate protein was passed down a column of Sephadex G-25 and the elution pattern compared with the elution positions of $^{3}$H-leupeptin and homogenate protein. Any shift in the elution profile of $^{3}$H-leupeptin towards the protein elution position in the mixture would indicate that leupeptin had become associated with the homogenate protein. The percentage of $^{3}$H-leupeptin that was spun down, with either cell debris or the TCA-precipitated fraction of a yolk-sac homogenate, was also determined.

To summarise, the following were investigated using $^{3}H$-labelled substrates:-

i) The uptake characteristics of $^{3}H$-leupeptin were compared with those of $^{3}H$-inulin (which was shown to be a suitable marker for fluid-phase pinocytosis). The effects of variation of leupeptin concentration (over the range 10µg-100µg/ml), of lowering the temperature of incubation (4°C - 37°C), and of a metabolic inhibitor on the rate of accumulation of $^{3}H$-leupeptin, were determined.

ii) Release of $^{3}H$-leupeptin and fluid-phase markers of different molecular mass was monitored in the presence and absence of ammonium chloride.

iii) Possible association between macromolecular components of the yolk-sac homogenate and $^{3}H$-leupeptin was investigated.

iv) Attempts were made to separate intact leupeptin from its putative degradation products (Appendix 4).

## 6.2. MATERIALS AND METHODS

### I. MATERIALS

**Equipment**

PD-10 Columns — Pre-filled with Sephadex-G25. Bed volume 8.5ml Pharmacia Ltd., Milton Keynes, Bucks., U.K.

**Reagents**

$^{3}$H-Leupeptin — The material used was synthesized by Dr.J. Powers and kindly donated to the author by Dr.R. Beynon, Dept. Biochemistry, University of Liverpool. It consisted of a total mass of 4mg, with an activity of 62µCi. A stock solution (1mg/ml in distilled water) was prepared and dispensed as 0.5ml aliquots for storage at -20°C. As required, this solution was diluted to give a 'working' stock solution (usually 25µg/ml), which was diluted 10-fold on addition to incubation flasks.

$^{3}$H-Inulin — Code TRA.324. Amersham International PLC, Bucks. The solid was dissolved in distilled water to give a 0.14mg/ml stock solution, which was stored in sealed ampoules at -20°C. This solution was generally diluted in medium 199 to 3.5µg/ml, before addition to incubation flasks to give a final concentration of 0.35µg/ml.

U-$^{14}$C-Sucrose — Code CFB.146. Amersham International PLC, Bucks. Diluted in water to give a 20µg/ml stock

solution, stored at-20°C. Stock solution generally diluted in medium, to 0.4µg/ml, before addition to flasks to give a final concentration of 0.04µg/ml.

Scintillation Fluid — Optiphase 'MP'. LKB, South Croyden, Surrey, U.K.

II. **METHODS**

6.2.1. Uptake of Radio-Labelled Markers

6.2.1a. **Uptake of $^{3}$H-Inulin**

Since the uptake of $^{3}$H-inulin had not previously been studied in the yolk-sac system, it was necessary to verify that the molecule was suitable for use as a marker of fluid-phase pinocytosis.

The methods of incubation of yolk sacs and of preparation of samples of medium and tissue for assay are described in full in Sections 2.2, 2.5, and 2.7c. Uptake was monitored both in the presence and the absence of 10% calf-serum in the medium. Uptake of $^{125}$I-PVP was monitored in matched control experiments (as described in Sections 2.2, 2.4a, and 2.7a) to ascertain the rate of fluid-phase pinocytosis.

Possible degradation of $^{3}$H-inulin was investigated by column chromatography. Yolk sacs (3) were incubated for 3h in medium 199 (7ml) containing $^{3}$H-inulin (0.7µg/ml) as described in Section 2.2, then rinsed in three changes of fresh warm medium. The yolk sacs were then re-incubated in fresh medium for 3h. Samples of the re-incubation medium, which would contain any degradation products, were loaded onto a gel chromatography column (PD-10) and eluted with water. A sample of inulin stock solution, that had not been incubated with yolk sacs, was passed down the same column, and the elution profiles compared.

The time-course of release of $^{3}$H-inulin-derived radioactivity from pre-loaded yolk sacs was also monitored, and compared with release of $^{125}$I-PVP. The experiment was carried out as described in Section 6.2.2. except yolk sacs were re-incubated in ammonium chloride-free medium only. The following modifications were necessary for the $^{125}$I-labelled substrate.

$^{125}$I-PVP was present in the loading medium at a concentration of about 35µg/ml. Yolk sacs were digested in NaOH (1M, 5ml/yolk sac) before assaying for radioactivity and protein, as described in Section 2.4a and 2.3. Samples (1.0ml) of medium were also assayed for radioactivity, as described in Section 2.4a. Quantitation of release of $^{125}$I-PVP was as described in Section 6.2.2., except correction for relative counting efficiency was unnecessary. Once these preliminary experiments comparing the characterisics of $^{3}$H-inulin uptake had been completed, $^{3}$H-inulin was used as a marker with which to compare accumulation of $^{3}$H-leupeptin.

6.2.1b. **Accumulation of $^{3}$H-Leupeptin**

Accumulation of $^{3}$H-leupeptin was monitored as described in Sections 2.2b and 2.5, and calculated as described in Section 2.7c. Uptake of $^{3}$H-inulin was monitored in matched experiments to indicate the rate of fluid-phase pinocytosis.

i) **Concentration Dependence:** The effect of leupeptin concentration on the rate of accumulation was determined over the range 3-100µg/ml. In experiments in which leupeptin was used at a concentration of 3, 5 or 10µg/ml, only $^{3}$H-leupeptin was added to the medium. In experiments with leupeptin at a concentration of 50 and 100µg/ml, $^{3}$H-leupeptin was at a final concentration of 10µg/ml, and unlabelled leupeptin at concentrations of 40 and 90µg/ml.

ii) **Temperature Dependence:** Accumulation of leupeptin was monitored at 4°C, 25°C, 30°C, 34°C and 37°C. (Uptake of $^{3}$H-inulin was monitored in matched experiments at the same temperature.) The experiments were performed as described in Chapter 2, except that the water bath in which the incubation flasks were placed was maintained at the above temperatures.

The normal equilibration period of approximately 10min was considered sufficient to permit the tissue to adjust to the lower temperature. For these experiments, $^{3}H$-leupeptin was used at a final concentration of 2.5µg/ml, and unlabelled leupeptin at 100µg/ml.

iii) **Effects of a Metabolic Inhibitor :** The rates of accumulation of $^{3}H$-leupeptin and $^{3}H$-inulin were monitored in the presence of rotenone ($10^{-5}M$). Experiments were carried out as described in Section 2.2b, except that rotenone was present in the incubation medium. $^{3}H$-Leupeptin or $^{3}H$-inulin was added to give final concentrations of 5 and 0.35µg/ml, respectively.

6.2.2. Release of Radiolabelled Substrates

6.2.2a. **Incubation Method.** Release of radioactivity derived from ingested $^{3}H$-inulin, $^{14}C$-sucrose, and $^{3}H$-leupeptin was monitored in the presence and absence of ammonium chloride (10mM).

Yolk sacs were loaded with substrate by incubating for 2-3 hours in flasks containing 3-5 yolk sacs in a final volume of 10ml medium. The final substrate concentrations during this period were: $^{3}H$-inulin, 1.4µg/ml; $^{14}C$-sucrose, 0.25µg/ml; and $^{3}H$-leupeptin, 5µg/ml. (Experimental details of yolk-sac incubation are given in Section 2.2.). After loading, the yolk sacs were rinsed in three changes of warm (37°C) fresh medium (approximately 20ml, 3 x 2min). The yolk sacs were then transferred to flasks containing warm, fresh, gassed, substrate-free medium (7-10ml) with or without ammonium chloride (10mM), and were re-incubated for a further 4 hours, (keeping the same 3-5 yolk sacs together throughout). After re-incubation the yolk sacs were rinsed in ice-cold saline, homogenized in water (1ml per yolk sac), and assayed for radioactivity and protein content as described in Sections 2.5 and

2.3. At intervals during the re-incubation, samples of medium (0.5ml) were removed from the flask for assay of radioactivity (as described in Section 2.5.) and the medium in the flask replenished with fresh warm medium, with or without ammonium chloride. The flasks were gassed (95%$O_2$: 5%$CO_2$) before commencement of the loading and re-incubation and after removal of each medium sample.

In one experiment, the amount of leupeptin-derived radioactivity released during each saline rinse was assayed in the presence and absence of cold leupeptin (50µg/ml), to determine whether $^3$H-leupeptin was displaced from cell-surface binding sites.

6.2.2b. **Quantitation of Release.** The amount of radioactivity (c.p.m.) released from the yolk-sac tissue was expressed in the same units as uptake (ie µl of medium per mg yolk-sac protein). This was then expressed as a percentage of the amount present in the yolk sac at the start of re-incubation.

The total amount of radioactivity released after a particular re-incubation period was calculated using the following equation (modified from Williams et al., 1975a):-

$$T_n = V.C_{i(i=n)} + x\sum_{i=0}^{i=n-1} Ci$$

Where $T_n$ = Total radioactivity (c.p.m.) released during the re-incubation period up to the time of the $n^{th}$ sample.

$V$ = Volume (ml) of medium during the re-incubation.

$C_i$ = Radioactivity (c.p.m) per ml re-incubation medium the $i^{th}$ sample, corrected for background and relative counting efficiency (see Section 2.5).

$x$ = Total volume removed during sampling (generally 2 x 0.5ml).

The total radioactivity released ($T_n$) was then expressed in terms of µl medium released per mg yolk-sac protein as follows:-

$$R_n = \frac{T_n}{M.P}$$

Where $R_n$ = Total volume (µl) of medium whose contained substrate was released from the yolk sac during the re-incubation period up to the time of the $n^{th}$ sample.

$M$ = Radioactivity per µl loading medium (c.p.m. corrected for background and relative counting efficiency).

$P$ = Protein content of the yolk sac (mg).

The accumulation (µl/mg) into the yolk sacs at the start of re-incubation was calculated as described in Section 2.7c, except the total radioactivity released from the yolk sac (ie $T_n$ where n = final sample) was added to the total radioactivity observed in the yolk sac after re-incubation. The amount of substrate released (ie $R_n$) was

then expressed as a percentage of the total accumulation.

6.2.3. Binding of $^{3}$H-Leupeptin to Yolk-Sac Homogenate Proteins

An investigation was made to determine whether or not $^{3}$H-leupeptin became bound to yolk-sac homogenate proteins. A yolk-sac homogenate was mixed with $^{3}$H-leupeptin and passed down a PD-10 gel-chromatography column. The elution profile was compared with that of homogenate protein and $^{3}$H-leupeptin. The experiment was carried out as follows.

A solution of leupeptin was prepared by adding 10µl of $^{3}$H-leupeptin solution (1mg/ml) to 1ml unlabelled leupeptin (50µg/ml). A sample (0.1ml) of this solution was added to 0.4ml distilled water, loaded onto the column and eluted with water, as described below. Yolk-sacs (3) were homogenized in 0.1% Triton X-100 (0.75ml) in a hand-held ground-glass-on-ground-glass homogenizer. Aliquots (0.5ml) of this homogenate were treated as follows:-

i) Duplicate samples were centrifuged (1000g, 20 min) and the supernatant decanted from the pelleted cell debris. The above leupeptin solution (0.1ml) was added to each supernatant, and the volume of each noted. An aliquot of this supernatant/leupeptin mixture (0.1ml) was taken for assay of total radioactivity, and the remaining volume adjusted to 0.5ml before loading onto the column. The sample was eluted with distilled water; 25 x 0.5ml fractions of eluant were collected. These were assayed for radioactivity.

ii) Leupeptin solution (0.1ml) was added to each of duplicate aliquots of homogenate, then the mixture centrifuged at 1000g for 20 minutes. The pelleted cell debris was resuspended in 0.5ml distilled water and assayed for radioactivity. The volume of the supernatant was noted, and a 0.1ml sample removed for

assay of total radioactivity. The remaining volume was adjusted to 0.5ml and the sample loaded onto the column and eluted as described above.

iii) 0.1ml of the leupeptin solution was added to duplicate aliquots of homogenate, then 0.5ml TCA (5%w/v) was added. The resulting precipitate was spun down (1000g, 20 min). The pelleted precipitate was re-suspended in 0.5ml distilled water and assayed for radioactivity. The volume of the soluble fraction was noted; 0.1ml of this solution was reserved for assay of radioactivity and 0.5ml was loaded onto the column and eluted as described above.

iv) One aliquot of the homogenate was centrifuged (1000g, 20min) and the volume of supernatant adjusted to 0.5ml. This was loaded onto the column and eluted as above, except that the eluant fractions were assayed for protein content (by the Folin assay) rather than for radioactivity.

The results were plotted as observed counts (c.p.m. per fraction) against fraction number. For the experiments described in parts (ii) and (iii), the percentage of leupeptin pelleted with the cell debris and TCA-precipitate during centrifugation was determined. The counts observed in the pellet and samples of supernatant assayed for radioactivity as described above, were normalized to correct for differences in counting efficiency. [The relative counting efficiency (with respect to water) of each sample was determined by comparing the observed counts in a "spike" of tritiated water added to samples of $^{3}$H-leupeptin-free pellet, supernatant and distilled water, as described in Section 2.5.] The amount pelleted was then calculated:-

$$\text{Percentage Pelleted} = \frac{\text{Counts in pellet (c.p.m., normalized)}}{\text{Counts in pellet (c.p.m., normalized)} + \text{counts in total volume of supernatant (c.p.m., normalized)}} \times 100$$

## 6.3. RESULTS

### 6.3.1. Uptake of $^{3}$H-Inulin: Assessment of Suitability for use as a Marker of Fluid-phase Pinocytosis.

The results in this section are those obtained when comparing uptake of $^{3}$H-inulin with that of the known fluid-phase marker of pinocytosis, $^{125}$I-PVP. In the absence of serum the mean Endocytic Indices (± S.D.) of $^{3}$H-inulin and of $^{125}$I-PVP were 3.53 (± 1.5 ) and 3.89 (± 1.8)µl/mg/h respectively. In the presence of 10% calf-serum the E.I.'s decreased to 3.18 and 2.81µl/mg/h respectively. Thus the Endocytic Indices of $^{3}$H-inulin and $^{125}$I-PVP were very similar, suggesting that uptake of $^{3}$H-inulin was by fluid-phase pinocytosis.

Both the standard volume and reduced volume methods of incubation were used in the absence of serum to monitor $^{3}$H-inulin uptake. In matched experiments, virtually no difference was observed in the mean E.I. of $^{3}$H-inulin using the different methods (2.39µl/mg/h for reduced volume; 2.33µl/mg/h for standard volume). The mean uptake value at each time point was calculated from a number of reduced volume uptake experiments, and the plot of mean uptake against time (Fig. 6.1) inspected for linearity. The plot is essentially linear, with an E.I. of 2.53µg/mg/h, (correlation coefficient 0.997). The standard deviation of points beyond 5h is high. (This was also observed for $^{125}$I-PVP uptake beyond 6h, in results described in Chapter 5.) Possible explanations for this are given in Section 6.4.2.

The possibility of intracellular degradation of $^{3}$H-inulin was investigated by column chromatography. The elution profile of $^{3}$H-inulin that had been released from loaded yolk-sacs (Fig. 6.2) can be compared with that of $^{3}$H-inulin that had not been incubated, (Fig. 6.3). The elution profiles are very similar, the majority of the

$^{3}$H-inulin passing down the column in the void volume.

The release of $^{3}$H-inulin and $^{125}$I-PVP from pre-loaded yolk-sacs was monitored in the presence and absence of serum (Fig. 6.4). Different batches of $^{3}$H-inulin were used in the presence and absence of serum. These results show several characteristic features.

i) The total amount of substrate released by 4.0 hours was greater in the absence of serum. This was particularly noticable for $^{3}$H-inulin.

ii) The total amount of $^{3}$H-inulin released by 4.0 hours was always greater than that of $^{125}$I-PVP, in matched experiments.

iii) Over the period 0.25-4h, the rate of release of $^{3}$H-inulin was greater than that of $^{125}$I-PVP in the absence of serum, but about the same in the presence of 10% serum.

### 6.3.2. Accumulation of $^{3}$H-Leupeptin

The rate of accumulation of $^{3}$H-leupeptin was compared with that of $^{3}$H-inulin, under various incubation conditions. The time-course of the uptake/accumulation (± standard deviation) for all $^{3}$H-inulin and all $^{3}$H-leupeptin experiments carried out at 37°C in the absence of serum (ie at various substrate concentrations, experiments not necessarily matched) is given in Fig. 6.5. The plot of uptake of $^{3}$H-inulin was approximately linear and, although the mean value of $^{3}$H-leupeptin accumulation gave a curved plot, the standard deviation about the mean was such that the time course of accumulation of $^{3}$H-inulin and $^{3}$H-leupeptin were indistinguishable.

**6.3.2a. Effect of Extracellular Leupeptin Concentration on the Rate of Accumulation of $^{3}$H-Leupeptin.** Uptake of a substrate by a mechanism such as adsorptive pinocytosis or facilitated membrane transport increases with increasing substrate concentration up to a

maximum rate, when the transport system becomes saturated. No further increase then occurs. If the concentration of labelled substrate is constant and only the unlabelled substrate concentration increases (as is often the case in experimental design) the E.I. of the substrate would appear to decrease with increasing concentration, as competition between labelled marker and unlabelled molecule occurs.

For non-saturable uptake mechanisms, eg fluid-phase pinocytosis or passive diffusion, the amount of substrate taken up (µg/ml tissue protein/h) will increase linearly with increasing concentration, thus the E.I. (µl/mg/h) will remain constant.

Table 6.1 reports the rate of accumulation and correlation coefficients calculated from the mean time-courses of accumulation of $^{3}$H-leupeptin at concentrations of 3, 5, 10, 50, and 100µg leupeptin/ml together with the Endocytic Indicies and correlation co-efficients of $^{3}$H-inulin (concentration 0.35 µg/ml) determined in matched experiments.

The rate of accumulation of leupeptin was typical of that of fluid-phase pinocytosis. There was no systematic change in the rate of accumulation with increasing concentration of leupeptin.

The time-course of accumulation of $^{3}$H-leupeptin at concentrations of 10µg/ml and 100µg/ml together with matched $^{3}$H-inulin controls, are shown in Figs. 6.6 and 6.7 respectively. The time-course of accumulation of each substrate in the matched experiments was virtually identical, further supporting the evidence for a lack of change in rate of accumulation with leupeptin concentration.

6.3.2b. **Effect of Temperature on the Rate of Accumulation of $^3$H-Leupeptin and $^3$H-Inulin.** The rates of accumulation of $^3$H-leupeptin (100µg/ml) and $^3$H-inulin (0.35µg/ml) were monitored at 4, 25, 30, 34 and 37°C. Figs. 6.8 and 6.9 show the time-courses of accumulation at each of these temperatures for $^3$H-leupeptin and $^3$H-inulin respectively. These plots indicate that the rates of accumulation of both $^3$H-inulin and of $^3$H-leupeptin were strongly temperature-dependent.

At 4°C, accumulation of both substrates was completely inhibited. Between 25 and 34°C, the rate of accumulation increased markedly. Accumulation at 34°C appeared to be indistinguishable from that at 37°C (the standard deviation at most points overlapped ).

The degree of scatter about the mean value of uptake was high, especially for incubation periods of 6 - 8 hours. For both substrates, the time-course of uptake started to level off at 37°C after 6h. Possible reasons for non-linear time-courses of accumulation are discussed in Section 6.4.2.

Because of this curvature, it was decided to calculate the rates of uptake/accumulation of the substrates over the initial 4h period of incubation only. These rates were determined for each individual uptake experiment, then the mean rate and standard deviation calculated for each temperature. The results are summarized in Fig. 6.10. Over the temperature range 25 - 37°C the mean rate of accumulation of each substrate increased in direct proportion to the temperature.

The temperature coefficient ($Q_{10}$) calculated from the gradient of the straight line drawn between the points was 1.92 for $^3$H-leupeptin and 2.59 for $^3$H-inulin. These are fairly typical of values for metabolic reactions, which generally have a $Q_{10}$ of about 2. An Arrhenius-type plot, of 1/T(K) against the mean natural

log of the rate of uptake/accumulation was non-linear for both $^{3}$H-leupeptin and $^{3}$H-inulin (see Fig. 6.11).

6.3.2c. **Effect of Pinocytic Inhibitors on $^{3}$H-Leupeptin and $^{3}$H-Inulin Accumulation.** Experiments were carried out using rotenone ($10^{-5}$M) as an inhibitor of pinocytosis. Accumulation of both $^{3}$H-inulin and $^{3}$H-leupeptin were strongly inhibited, see Fig. 6.12.

6.3.3. Release of Radiolabelled Substrates from Loaded Yolk Sacs: Effect of Ammonium Chloride

Release of the fluid-phase pinocytic markers $^{3}$H-inulin (mol. mass 5,200) and $^{14}$C-sucrose (mol. mass 342) from loaded yolk sacs was monitored in the presence and absence of ammonium chloride (10mM). Release of radiolabel from yolk sacs loaded with $^{3}$H-leupeptin (ie release of radiolabelled intact leupeptin and/or degradation products) was also monitored, for comparison with both the rate of release of fluid-phase markers and the rate of loss of leupeptin from yolk-sacs observed in Section 5.3.4 and 5.3.5.

In preliminary experiments the rate of uptake of $^{14}$C-sucrose was determined, to confirm the previously reported rate and mode of uptake of this marker in yolk sacs (Roberts et al., 1977). The time-course of uptake is shown in Fig. 6.13. The plot was linear over the first 4h of incubation, then a slight decrease in the rate of accumulation occurred. (Possible explanations for this observation are given in Section 6.4.2.)

The time-courses of release of $^{14}$C-sucrose, $^{3}$H-inulin and $^{3}$H-leupeptin (and/or degradation products) are shown in Figs. 6.14, 6.15 and 6.16 respectively.

The total percentage of substrate released by 4h, when the

tissue was incubated in serum-free medium, varied greatly between markers: $^{14}$C-sucrose (25%), $^{3}$H-inulin (15%), and $^{125}$I-PVP (5%). [Release of $^{3}$H-leupeptin should not be compared directly with these results because it was not established whether the material released was intact leupeptin or degradation products (in which case the amount released would not depend on the membrane permeability towards leupeptin). The total quantity of $^{3}$H-leupeptin-derived radiolabel released after 4h was about 28%.]

Release of $^{14}$C-sucrose (Fig. 6.14) proceeded rapidly over the first hour and release was slightly more rapid in the presence of ammonium chloride. The rate of release then slowed considerably; after about 2.5h no further substrate was released. The amount of radioactivity that had been released after this period in the presence of ammonium chloride was virtually the same as that released in its absence.

The pattern of release of $^{3}$H-inulin was slightly different (Fig. 6.15). The percentage released over the first hour of re-incubation rose only slightly (from about 8 to 13%) and no marked difference was apparent between yolk sacs re-incubated with or without ammonium chloride. The rate of release between 1-4h was very slow (about 0.9%/h), but the amount released did not reach a constant value (c.f. results for $^{14}$C-sucrose).

Release of $^{125}$I-PVP in the presence and absence of ammonium chloride was investigated in Chapter 5 (results in Section 5.3.4b; see Fig 5.11) and results were found to be similar whether the increase in radioactivity in the medium or the decrease in radioactivity in the tissue was monitored. No large initial release of this marker was observed, only about 5% of the tissue-associated $^{125}$I-PVP was released by the end of the re-incubation period. No difference was observed in results obtained in the presence or

absence of ammonium chloride.

Fig. 6.16 shows the release of radiolabel from yolk sacs loaded with $^{3}$H-leupeptin. Unlike the results observed in Section 5.3.4 (in which loss of active leupeptin was monitored) the release pattern of $^{3}$H-label was the same whether or not ammonium chloride was present. A rapid initial release (over the first hour of reincubation) was followed by a slightly slower rate of release that continued over the entire reincubation period. (A constant plateau was never reached.) The rate between 1-4h (about 3.5% per h) was greater than that observed for any of the other substrates.

### 6.3.4. Binding of $^{3}$H-Leupeptin to Yolk-Sac Homogenate Proteins

In Section 5.4.2. it was suggested that a possible explanation for the poor recovery of leupeptin from yolk-sac homogenates was that the leupeptin became bound to yolk-sac protein(s) (eg cathepsins B and L, to which it binds tightly) and was co-precipitated with them on addition of TCA. In this chapter it was hoped to detect any such protein-bound leupeptin by differentiating between protein-bound and free $^{3}$H-leupeptin in a yolk-sac homogenate/$^{3}$H-leupeptin mixture, using a gel-chromatography column. The TCA-soluble fraction of homogenate/$^{3}$H-leupeptin was also eluted from the column to determine whether any changes in the elution characteristics of $^{3}$H-leupeptin occurred after treatment with TCA.

Gel-chromatography on Sephadex separates molecules chiefly according to molecular size, although ionic charge and hydrophobicity may also affect the elution position, particularly if water is used as the eluant. (Aromatic and positively-charged molecules are retained on the gel, negatively-charged molecules are excluded.) The column used for these experiments contained Sephadex G25, which has a fractionation range of about 1,000 - 5,000 for peptides and globular

proteins. Free $^{3}$H-leupeptin, since it is below this fractionation range, would therefore be expected to elute with an elution volume approximately equal to the bed volume. Cell proteins would be expected to elute in the void volume, provided their molecular mass was greater than 5,000. Small polypeptides may elute anywhere between the void volume and the bed volume.

The eluant collected during sample loading was termed the first fraction, and each fraction volume was 0.5ml. Dextran-blue (a high mol. mass dye) was eluted in fractions 8 to 10 indicating that the void volume of the column was about 4ml. The bed volume of the column was reported to be 8.0ml.

Fig. 6.17 shows a typical elution profile of a sample of $^{3}$H-leupeptin that had not been mixed with a yolk-sac homogenate. An elution peak occurred at a volume of 8ml, (ie equal to the bed volume). A slight 'shoulder' was observed before the main peak, at an elution volume of about 5ml. This could represent higher-molecular-mass impurities or possibly forms of leupeptin with different hydration (eg cylic form or alcohol form - see Fig 1.1). The nature of the material in this subsiduary peak was not determined.

Fig. 6.18 shows a typical elution profile of yolk-sac proteins, as determined by the Folin assay. [Colour development with the Folin-Ciocalteu's phenol reagent is greatest for proteins, but peptides and aromatic amino acids will also react, Lowry *et al.*, 1951.] Two major peaks occurred, at elution volumes of 4.5 and 10ml, corresponding approximately to the void volume and a volume greater than the bed volume. Some Folin-positive material also eluted in volumes between these peaks. The elution profile indicates that the yolk-sac homogenate contained a range of proteins with different molecular mass. Those with a molecular mass over 5,000 (which

includes cathepsin B and L, mol. mass 25,000 and 24,000 respectively; Barrett & McDonald, 1980) should have eluted in the void volume. Proteins and polypeptides with a molecular mass between 1,000 - 5,000 eluted between the void and bed volume. (The resolution of the PD-10 columns was insufficient to distinguish individual proteins over this fractionation range.) Folin-positive amino acids and peptides with a molecular mass less than 1,000 generally elute either with or after the bed volume, depending on the gel-solute interactions, and must have given rise to the second observed peak. Comparatively few Folin-positive compounds eluted in the same position as $^{3}$H-leupeptin, ie with an elution volume of 5 to 8ml.

For experiments in which $^{3}$H-leupeptin was added to a whole yolk-sac homogenate that was then centrifuged to remove cell debris, a typical elution profile of the supernatant was as shown in Fig. 6.19. The main peak occurred at an elution volume of 7.0ml, with a small subsiduary peak at 4.5ml. This elution profile is similar to that of 'pure' $^{3}$H-leupeptin, except that the position of the peaks was shifted, by about 1ml, towards that expected for larger/more positively-charged molecules. The elution bands did not correspond to either of the main protein elution bands, therefore it seems unlikely that the shift in the leupeptin elution position was caused by leupeptin becoming bound to a homogenate protein. (In particular, these results indicate that leupeptin did not elute with cathepsins B and L in the void volume.)

The percentage of $^{3}$H-leupeptin that became trapped within the pelleted cell debris could be determined by counting samples of the supernatant and pellet for radioactivity. (It was necessary to correct the observed count for background and relative counting efficiency). About 10% of the added radioactivity became associated with the pelleted cell debris.

A typical profile of $^{3}$H-leupeptin that was added to the supernatant of a yolk-sac homogenate (after cell debris had been centrifuged down) is shown in Fig. 6.20. The elution pattern was virtually identical to that shown in Fig.6.19 (for which $^{3}$H-leupeptin was added before centrifugation).

Fig. 6.21 shows the elution profile for the TCA-soluble fraction of a yolk-sac homogenate to which $^{3}$H-leupeptin had been added. A single elution peak occurred at a volume of 7ml, which is at a similar position to that of the main elution peak observed in Figs. 6.19 and 6.20. However in the presence of TCA there was no subsiduary peak. This suggests that the material that gave rise to the subsidiary peak was precipitated, or co-precipitated, by TCA. Since the nature of the material was unknown, no definite correlation could be made between loss of this material and poor recovery of leupeptin activity after treatment with TCA, but it seems unlikely that loss of the quantity of material present in the subsidiary peak could account for the observed loss of about 50% of leupeptin activity. (The material may have been a tritiated impurity that was precipitated by TCA.)

The percentage of radioactivity that became associated with the pelleted TCA precipitate was about 20%, which although fairly high, does not account for all of the loss of leupeptin activity.

## 6.4. DISCUSSION

### 6.4.1. Use of Radiotracers to Monitor Endocytosis

The widespread use of radiolabelled substrates in the study of endocytosis results from the many advantages inherent in their use. Some of these are briefly listed below.

Firstly, detection of the radiolabel is a rapid and experimentally easy method, and the counting process is generally automated. This is a major advantage when monitoring a large number of samples.

Secondly, it is usually possible to detect very small quantities of radiolabelled substrate, especially if both the specific radioactivity (ie d.p.m. per unit quantity of substrate) and the efficiency of detecting the emitted radiation are high. Only tracer quantities of radiolabelled substrate need be used, therefore uptake of low concentrations of substrates can be monitored.

Thirdly, the radiolabel remains detectable even if the substrate becomes degraded within the cell. The amount of radioactivity within a tissue/cell incubation system will not change, but the distribution between tissue and medium or between different subcellular compartments may do so. The proportion of intact to degraded molecules may well differ throughout the course of an experiment, and with suitable analytical methods it is possible to monitor such changes, and hence follow the intracellular fate of a substrate. However if no convienient method exists to separate the intact compound from its metabolites, problems may arise in interpretation of data.

Fourthly, use of radiolabelled tracers has advantages over methods of quantitating endocytosis based on morphometric analysis. The advantages and disadvantages of the use of radiolabelled markers

as opposed to morphometric techniques are discussed by Pratten et al. (1980) and Silverstein et al. (1977).

Disadvantages inherent in the use of radiolabelled markers depend to a large extent on the type of radioisotope used.

$^{125}I$-labelled compounds have been used in the work reported in the earlier chapters of this thesis; some problems associated with their use are discussed below.

i) There is a possibility of a difference in biological behaviour between labelled and non-labelled material, arising as a result both of incorporating a "foreign" iodine atom, and of oxidation/reduction reactions that may occur during labelling.

ii) Iodinated compounds have limited 'shelf-life'. ($^{125}I$ has a half-life of sixty days, and loss of radioiodide from the labelled molecule, or denaturation, or aggregation of material during storage may cause problems.)

These problems are offset by the ease of sample preparation for gamma-counting, and the greater count rate produced by $^{125}I$ compared with equal gram-quantities of $^{3}H$ and $^{14}C$. A further advantage in some experiments using $^{125}I$-labelled proteins, is the lack of re-incorportation of the labelled degradation product $^{125}I$-Tyr. Advantages and disadvantages inherent in the use of $^{125}I$-labelled products are reviewed by Bolton (1977).

Beta-emitting radioisotopes were used extensively in the work reported in this chapter. Several problems are associated with their use:-

i) $^{3}H$ may dissociate from the labelled molecule; the likelihood of dissociation depends on the position of the labelled atom. Generally labile $^{3}H$-hydrogen atoms are removed from the parent molecule immediately after synthesis. However, slow exchange of

non-labile $^{3}H$-hydrogen atoms with hydrogen atoms from the solvent may occur during storage, producing $^{3}H_2O$ as an impurity. This problem does not arise with $^{14}C$-labelled compounds, as carbon atoms do not exchange with the solvent.

ii) $^{3}H$- or $^{14}C$-labelled components of a degradable biopolymer are likely to become utilized by the cell in biosynthetic pathways, due to their being structurally identical to unlabelled components. [This problem may be overcome by ensuring that a large excess of unlabelled component(s) are present during a 'chase' period.]

iii) Beta radiation (and, in particular, $^{3}H$-beta-emissions) are not very penetrating, therefore the isotope must be in close contact with the scintillator for transmission of radiation energy. Ideally the sample to be counted should be dispersed evenly throughout the scintillation fluid as a homogeneous solution or heterogeneous suspension or emulsion. This may require careful and time-consuming sample preparation.

iv) Beta radiation is susceptible to quenching, in which energy is absorbed by non-scintillant components in the sample mixture. Colour quenching occurs in samples containing chromophores, and chemical quenching occurs in samples containing, for example, proteins.

The latter two factors may give rise to a poor counting efficiency for beta radiation. Many methods have been developed to maximize the detection efficiency and/or correct for quenching (eg see Peng, 1977). Those relevant to the yolk-sac system are discussed below.

Several methods of preparation of yolk-sac tissue were assessed for reproducibility and recovery, the results are given in Appendix

3. None of the methods used was ideal. Homogenization in water was used for experimental tissue because this gave the most reproducible results. Preparation of tissue for radioassay is discussed by Silverman et al. (1985).

As pointed out in Section 2.7c, the number of counts observed in samples of medium 199 and of yolk-sac tissue are not immediately comparable, because the counting efficiency of each type of sample is different. The counts observed in a 'spike' of radiolabelled compound in both medium 199 and tissue homogenate was compared with that observed in water, and the counting efficiency of each type of sample relative to water determined. The observed counts were then normalized using this relative counting efficiency. These normalized counts could be compared directly to calculate the volume of incubation medium whose contained substrate was associated with the tissue, (ie µl/mg yolk-sac protein).

The use of $^{3}H$-labelled leupeptin enabled the peptide to be detected reliably at very low concentrations, such as those present in yolk sacs when using low concentrations of substrate in the medium, or short incubation times, or when inhibitors of uptake were present. Also, less tissue was required at each time point to obtain sufficient $^{3}H$- leupeptin to assay.

### 6.4.2. Linearity of Accumulation

Many of the time-courses of accumulation determined during experiments reported in this chapter showed a tendency towards non-linearity. The rate of accumulation decreased with time, suggesting the approach of an intracellular steady-state. Such non-linearity was observed when accumulation of $^{3}H$-leupeptin, $^{14}C$-sucrose, and in some experiments, $^{3}H$-inulin, was monitored.

To avoid repetition in the various sections of this chapter, possible general causes of such non-linearity will be discussed below, together with the most likely explanation for each particular substrate. There are three main causes possible:-

i) The tissue viability may decrease during the incubation period, giving rise to a decreased rate of uptake and possibly an increased rate of release (due to increased cell lysis). Tissue was incubated in a small volume of serum-free medium for long periods of time, hence viability of the tissue may have been lowered towards the end of the incubation period. A high standard deviation about the mean accumulation value suggests reduced viability since individual tissues may well have different survival times. Such variability was observed for all substrates (ie $^{3}$H-leupeptin, $^{3}$H-inulin, $^{14}$C-sucrose and $^{125}$I-PVP).

ii) Release of intact substrate may occur during the incubation period, either by rapid exocytosis or by direct membrane permeation. The only mechanism of release for markers of pinocytosis such as $^{125}$I-PVP and $^{14}$C-sucrose (other than during cell lysis), is by exocytosis. Rates of exocytosis reported in the literature vary considerably. Some, but not all reports suggest that exocytosis is quantitatively significant during short-term tissue incubations and is sufficient to give rise to non-linearity in the time-course of accumulation (eg Besterman et al., 1981). However, in yolk sacs, the rate of exocytosis (as determined by release of $^{125}$I-PVP) appears to be slow, and is not sufficient to cause non-linearity of the time-course of accumulation in standard volume cultures (eg Ibbotson & Williams, 1979). The membrane permeability of yolk-sac membranes towards $^{3}$H-leupeptin and $^{3}$H-inulin was not known, therefore

release by direct membrane permeation of the intact molecule could not be dismissed as a possible cause of any non-linearity in the plot of accumulation against time. However, it was considered unlikely that $^{3}$H-inulin could pass through membranes intact, due to it large size and on consideration of the results of Bowers & Olszewski (1972), who found that $^{3}$H-inulin did not permeate through the plasma membrane of Acanthamoeba.

iii) Intracellular degradation of the substrate to yield membrane-permeable degradation products that are released from the tissue. Intracellular degradation of a substrate followed by release of membrane-permeable degradation products is often observed to give rise to an intracellular steady-state concentration of intact substrate plus degradation products. This occurs within 1-2h for protein substrates in rat yolk sacs (eg Williams et al., 1975b; Livesey & Williams, 1981). Intracellular degradation of $^{3}$H-leupeptin was considered to be quite probable (yolk-sac homogenate mediated loss of leupeptin activity was noted in Chapter 4, and degradation of leupeptin was reported by Beynon et al., 1981). $^{3}$H-Inulin and $^{14}$C-sucrose are not generally considered to be degradable intracellularly, hence were not expected to be released by this mechanism. However, release from yolk-sac tissue of radiolabel derived from these markers was found to occur at a greater rate than release of $^{125}$I-PVP (which is thought to occur by cell death alone). This suggests that either limited intracellular hydrolysis of $^{3}$H-inulin and $^{14}$C-sucrose occurs within yolk sacs, or the substrate preparations used contained readily hydrolysable radiolabelled contaminants, or the substrates stimulated the rate of exocytosis. Any one of these effects could lead to an increased rate of release and hence a non-linear time-course of

accumulation. Isolation and identification of the released radiolabelled products would be necessary to distinguish between these possibilities.

Hence for each substrate the following possible explanations for non-linarity of accumulation by the tissue could apply:-

**$^{125}$I-PVP.** The only likely reason for any non-linearity is reduced viability of the tissue, since abundant evidence exists to indicate that $^{125}$I-PVP is not rapidly released from yolk sacs and is not degraded within them. The time-course of uptake of $^{125}$I-PVP showed little tendency towards non-linearity, although the standard deviations about the mean for time points above 4h were large.

**$^{3}$H-Inulin and $^{14}$C-Sucrose.** In addition to reduced viability other explanations of the observed non-linearity are plausible. Radiolabel was released into the medium more rapidly for these substrates than for $^{125}$I-PVP, suggesting either stimulation of exocytosis or intracellular degradation of the substrate and/or radiolabelled contaminants to produce and to release degradation products. Substrate degradation would be expected to be limited in extent, since both markers are reported to be non-degradable. Release of either marker by direct membrane permeation was not expected, on consideration of previous reports (eg Bowers & Olszewski, 1972; Besterman *et al.*, 1981).

**$^{3}$H-Leupeptin.** Non-linearity in the time course of accumulation of this substrate could have been caused by any of the above explanations.

6.4.3. $^{3}$H-Inulin

The uptake kinetics of $^{3}$H-inulin were investigated to confirm whether this marker behaved in the same way as established markers of fluid-phase pinocytosis in the rat yolk-sac system.

Many criteria must be met before the mode of uptake of a molecule can be attributed to fluid-phase pinocytosis, eg see Pratten et al. (1980); Ose et al. (1980); Steinman & Cohn (1972); Steinman et al. (1974); Besterman et al. (1981). Briefly the various requirements of fluid-phase (as opposed to adsorptive) pinocytic markers are:-

i) Uptake should occur at the same minimum rate observed for other fluid-phase markers in that cell system.

ii) The rate of uptake (µg/h per mg tissue) should increase in a direct proportion to the extracellular concentration of substrate. (The E.I. expressed in terms of µl/mg/h, should remain constant.)

iii) The rate of loss of the substrate should be low, with no substantial, readily releasable component.

iv) The substrate should not associate with the cell/tissue at low temperatures or in the presence of pinocytic inhibitors.

Ideally, all these criteria should be established before uptake of a macromolecular substrate is claimed to occur via fluid-phase pinocytosis. Experiments in this section were therefore designed to assess whether they applied to the behavior of $^{3}$H-inulin in rat yolk-sac tissue.

The rate of accumulation of $^{3}$H-inulin was virtually identical to that of $^{125}$I-PVP, an established fluid-phase marker in the yolk-sac system. The time-course of accumulation was approximately linear, at least over the initial six hours of incubation. Only a very small concentration range of $^{3}$H-inulin was tested, so nothing conclusive

can be reported on the concentration dependence of the rate of accumulation.

The time-course of release of $^{3}H$-inulin was difficult to interpret. Both the amount and the rate of release of $^{3}H$-inulin were greater than that of $^{125}I$-PVP; the difference was particularly noticable in the absence of serum. [The effect of serum on release of substrates is not well documented, although some reports mention that the presence of serum (Ibboson & Williams, 1979) or concentration of serum (Dean & Jessup, 1982) has no effect on exocytosis.] A slight increase in the amount of substrate released in the absence of serum might be expected, since cell viability is slightly lowered, therefore cell death with consequent release of all cell contents, might occur to a greater extent. It is possible that the greater release of $^{3}H$-inulin compared with $^{125}I$-PVP was due to limited intracellular degradation of that molecule or of radiolabelled contaminants, which would also explain the tendency towards a decrease in the rate of accumulation on prolonged incubation, see Section 6.4.2. (Thus a greater amount of impurities in the different batch of $^{3}H$-inulin used in the absence of serum would explain the increased rate of release.) However, no impurities or lower molecular mass degradation products were observed when unincubated $^{3}H$-inulin and radiolabel released from yolk sacs loaded with $^{3}H$-inulin were passed down a Sephadex G-25 column.

The evidence presented in this section, coupled with the previously reported data on $^{3}H$-inulin (Bowers & Olszewski, 1972) strongly suggests that uptake of $^{3}H$-inulin by rat yolk sacs is via fluid-phase pinocytosis.

### 6.4.4. Accumulation of $^{3}$H-Leupeptin

The results discussed in this section refer only to the net accumulation of radiolabel within tissue, which does not necessarily correspond to the total uptake of leupeptin. However the rate of tissue accumulation of radioactivity is a good approximation to the total rate of uptake, provided the rate of release is not too great. (Release of leupeptin-derived radioactivity from yolk sacs is discussed in Section 6.4.5).

Experiments were designed to distinguish between possible routes of entry of leupeptin into the cell, ie passive diffusion, facilitated transport, active transport, fluid-phase pinocytosis, and adsorptive pinocytosis.

Measuring the rate of accumulation for increasing substrate concentrations should allow distinction between facilitated transport, active transport, or adsorptive pinocytosis (which are all saturable), and passive diffusion, or fluid-phase pinocytosis (which are not saturable). Accumulation of $^{3}$H-leupeptin over the concentration range 3-100µg/ml did not appear to show saturation kinetics. (No systematic change in the rate of accumulation of $^{3}$H-leupeptin with increasing concentration occurred). The time-course of accumulation at each concentration of leupeptin was indistinguishable from that of matched $^{3}$H-inulin controls.

In principle, metabolic inhibitors should permit differentiation between passive diffusion or facilitated transport (which do not require energy and are thus not inhibited) and active transport or pinocytosis (which are energy dependent, and are thus inhibited by metabolic inhibitors). However some metabolic inhibitors may have unwanted side effects; any changes in either the cell membrane or the substrate itself in the presence of inhibitor may affect both passive and facilitated transport. The metabolic inhibitor rotenone (which

inhibits the electron transport chain thus lowering cellular ATP levels) strongly inhibited $^{3}$H-leupeptin accumulation (Fig. 6.12). This inhibitor is not reported to affect energy-independent processes suggesting that accumulation of leupeptin requires energy.

The temperature dependence of a metabolic reaction is often given in terms of the temperature co-efficient ($Q_{10}$), which is the relative increase/decrease in the rate on raising/lowering the temperature by 10°C. The $Q_{10}$ value gives an indication of the activation energy of the transport mechanism (processes with a high activation energy will have a high $Q_{10}$ value). It was hoped that the temperature dependence of the rate of accumulation might allow differentiation between uptake via permeation and pinocytosis.

The $Q_{10}$ of permeation of different substrates across a specific plasma membrane will depend on the oil/water partition coefficient, molecular size, degree of hydration etc. of the substrate. Facilitated and active transport usually have a $Q_{10}$ greater than that which might be expected from the characteristics of the substrate if passive diffusion were the method of uptake, but this is not sufficiently reliable to be a diagnostic test. Membrane transport processes and permeability are generally affected by the transition temperature of the membrane (ie the temperature at which the lipids in the membrane start to crystallize, which depends on the lipid composition). The $Q_{10}$ values (and activation energy) of transport differ above and below this temperature, and membrane permeability shows a maximum at this temperature (Bach, 1983). Some exceptions are known, eg Racker & Hinkle (1974) suggest that transport by membrane pores may not change at the transition temperature.

Several reports have been made on the temperature dependence of pinocytosis, though some observations differ in detail. Most workers have found that pinocytosis is inhibited at and below about 15°C,

which corresponds roughly to the transition temperature of many membranes (eg Dunn et al., 1980; Shirazi et al., 1982; Duncan & Lloyd, 1978; Mahoney et al., 1977). However, Silverstein et al. (1977) and Ose et al. (1980) found no critical thermal transition point, pinocytosis continued at a rate directly proportional to temperature over the range 2-38°C. In general, above about 15°C the $Q_{10}$ and activation energy of fluid-phase pinocytosis is constant up to 38°C; the $Q_{10}$ is in the region of 2.8 (Shirazi et al., 1982; Pratten & Lloyd, 1979; Mahoney et al., 1977). The $Q_{10}$ of receptor mediated pinocytosis is often reported to show an inflection point at about 20°C, below which the $Q_{10}$ and activation energy of uptake is considerably increased (eg Weigel & Oka, 1981).

In yolk sacs, pinocytosis was totally inhibited at 20°C (Duncan & Lloyd, 1978). Unlike the results of Shirazi et al. (1982) an inflection point was observed at 30°C for fluid-phase pinocytosis, using $^{125}$I-PVP as a marker. The activation energy (and hence $Q_{10}$) above this temperature was greater than that below it (Duncan, 1978). Above 30°C, the $Q_{10}$ was 7.05, which is considerably higher than those reported for pinocytosis and other membrane transport mechanisms in other cells and tissues at temperatures between 30-37°C. Hence in rat yolk sacs it was just possible that the temperature dependence of uptake could be used to distinguish between pinocytosis and other mechanisms of transport.

However, temperature dependence of accumulation of neither $^{3}$H-leupeptin nor $^{3}$H-inulin concurred with that reported by Duncan (1978). The rate of uptake of the marker $^{3}$H-inulin decreased in direct proportion to temperature (with no discontinuity at 30°C). The $Q_{10}$ was 2.59, which is similar to those reported in other systems. The temperature dependence of $^{3}$H-leupeptin was similar to that of $^{3}$H-inulin except that the $Q_{10}$ was 1.92 which is slightly

lower than most reported values for pinocytic markers. Temperatures between 4-25°C were not tested therefore it is not possible to determine whether a transition temperature was apparent between these temperatures. It is likely that a transition occurred at or around 25°C, since uptake was almost fully inhibited at this temperature.

Arrhenius plots are frequently used to calculate the activation energy of transport processes. A discontinuity in the Arrhenius plot signifies a change in the activation energy and is observed, for example, when diferent processes become rate-limiting in pinocytosis (eg Wiegel & Oka, 1981) and/or at the membrane transition temperature (Bach, 1983). Although no clear discontinuity was observed between 25-37°C for the uptake of $^{3}$H-inulin and $^{3}$H-leupeptin the Arrhenius plot between these temperatures was not linear. A more informative plot may have been obtained if a greater range and larger number of temperatures had been used.

### 6.4.5. Release of Radiolabelled Substrates from Yolk-sac Tissue

It was hoped that the results from these experiments would provide information on the following points.

i) The mode(s) of release of $^{3}$H-leupeptin-derived radioactivity (by comparison with the release of pinocytic markers that are thought to be released only by exocytosis or cell death).

ii) The effect of $NH_4Cl$ on the release of $^{3}$H-leupeptin-derived radioactivity and pinocytic markers of various sizes, in order to further investigate the effect of $NH_4Cl$ on loss of leupeptin activity observed in Section 5.3.5.

### 6.4.5a. Mode of Release of $^{3}$H-Leupeptin

There are several ways by which an internalized substrate can be released from cells, depending on the nature of the substrate. Non-degradable molecules that are incapable of crossing cell membranes are released only by exocytosis and/or cell death. Substrates that are able to cross cell membranes are also released by direct permeation across the plasma membrane. Degradable molecules, even if incapable of crossing membranes intact, may be broken down into fragments that can be released by permeation. Different kinetics of release would be expected for these different types of substrate.

Markers of pinocytosis such as $^{3}$H-inulin, $^{14}$C-sucrose and $^{125}$I-PVP are thought to be released from cells via cell death or exocytosis alone. If the rate of release of $^{3}$H-leupeptin-derived radioactivity was found to be greater than the rate of release of these markers, $^{3}$H-leupeptin-derived label must be released by permeation, either as intact leupeptin or following degradation to membrane-permeable fragments.

Before discussing the patterns of release observed for the above substrates, it is pertinent to review some of the current literature on the expected kinetics of exocytosis of non-membrane-permeable, non-degradable substrates. Such substrates are generally released in a rapid initial burst followed by a slower constant rate of release.

In yolk sacs, Williams *et al.* (1975a) and Roberts *et al.* (1977) proposed that the initial burst of release was caused by dissociation of substrates trapped in extracellular sites such as the microvillus surface, open pinocytic channels and possibly intercellular spaces. The percentage of substrate released during this phase was not reproducible, the duration of which was about 1h. The slower, linear release phase was attributed to release of intracellular substrate by

cell lysis and possibly exocytosis.

However, other workers have proposed alternative mechanisms to account for the different release phases. Besterman et al. (1981) monitored the release of $^{14}$C-sucrose from macrophages and fibroblasts over a period of two hours. They proposed that the observed rapid initial release was caused by release of substrate from a small intracellular compartment with a short half-life, and the subsequent slower release phase by turnover of a larger compartment with a long half-life. The duration of each phase and percentage of substrate released depended on the loading period. Dean & Jessup (1982) characterised a linear release phase of $^{14}$C-sucrose from fibroblasts that occurred after 4h, and suggested that this release may reflect membrane recycling.

In the work reported in this thesis the release patterns of $^{3}$H-inulin and $^{125}$I-PVP (Sections 5.3.4, 6.3.1, and 6.3.3) were as expected for non-degradable pinocytic markers, ie an initial rapid release was followed by a slower, approximately linear, release. The percentage of $^{3}$H-inulin released during the initial phase was much higher than that of $^{125}$I-PVP. This may have been caused by the smaller $^{3}$H-inulin molecules becoming more readily trapped at extracellular sites. ($^{3}$H-Inulin is known to pass through tight junctions in liver cells, Lowe et al., 1985, thus may have greater access to intercellular spaces.) Possible causes for the slightly greater rate of release of $^{3}$H-inulin compared with $^{125}$I-PVP during the linear release phase are discussed in Section 6.4.2.

The release pattern of $^{14}$C-sucrose differed from that expected for non-degradable pinocytic markers and from that previously reported for $^{14}$C-sucrose in yolk-sac tissue (Roberts et al., 1977). The reason for this anomaly was not investigated, though it may have been caused by release of intact $^{14}$C-sucrose or degradation products

of $^{14}$C-sucrose (or $^{14}$C-sucrose contaminants) via membrane permeation.

The release pattern of radiolabel from $^{3}$H-leupeptin-loaded tissue was similar to that of $^{125}$I-PVP and $^{3}$H-inulin (a rapid initial burst was followed by a slower linear rate of release), except that the percentage of radioactivity released during the burst and the rate of release during the linear phase were greater than those of $^{3}$H-inulin and $^{125}$I-PVP. The initial burst of release may have been caused by the release of leupeptin that had become trapped at extracellular sites of the yolk sacs. (Adsorbtion of leupeptin to the plasma membrane was not thought likely because $^{3}$H-leupeptin was not displaced from the yolk sac during a 3x2 min rinse period in the presence of cold leupeptin.) The greater rate of release of $^{3}$H-label during the linear release phase could be caused by a slow release of intact leupeptin or leupeptin degradation products from the tissue by membrane permeation in addition to release by cell lysis or exocytosis. However, further evidence is required on the nature of the $^{3}$H-label released during the different phases before making any firm conclusions on the mechanism(s) of release of leupeptin.

### 6.4.5b Effect of Ammonium Chloride on the Release of $^{3}$H-Leupeptin Derived Radioactivity: Comparison with Pinocytic Markers

In the experiments reported in this section, the effect of ammonium chloride on the release of pinocytic markers of various molecular sizes and on the release of $^{3}$H-leupeptin-derived label from loaded yolk sacs was determined to try to explain the increased loss of active leupeptin discussed in Section 5.4.2.

The effect of ammonium chloride on the vacuolar system of cells is well documented. Cells become highly vacuolated (Seglen & Reith, 1976; Ohkuma & Poole, 1981). Many membrane fusion processes are inhibited, eg endocytosis, vesicle-lysosome fusion. Exocytosis is

also inhibited by up to 25%; the dose-response curve is biphasic, with maximal inhibition occurring at lower concentrations (Dean & Jessup, 1982). A review of the effect of amines on mammalian cells is given by Dean et al. (1984).

Unfortunately, little is known about the effects of ammonium chloride on membrane permeability or on membrane transport systems. The lysosomal membrane must expand to accommodate the increased lysosomal volume, which may result in the membrane becoming more porous. Also, it is possible that lysosomal membrane transport mechanisms may rely on the maintanance of a particular pH within the lysosome and thus would not operate normally in the presence of ammonium chloride.

Release of $^{125}$I-PVP and $^{3}$H-inulin were unaffected by ammonium chloride (10mM), (which suggests that either exocytosis was not inhibited in the yolk sac or the substrates were not released by exocytosis). No increase in cell lysis or in plasma membrane permeability, to molecules with a mol. mass >5,000 (detectable as an increased rate of substrate release) was thus apparent. The initial rate of release of $^{14}$C-sucrose was slightly increased, but because this substrate behaved anomolously in the absence of ammonium chloride, no conclusions can be drawn on the effect of ammonium chloride on its release.

Ammonium chloride did not increase the release of $^{3}$H-leupeptin. This suggests that the enhanced loss of leupeptin activity discussed in Section 5.4.2. was not due to release of intact leupeptin from the tissues. The loss may have been caused by leupeptin inactivation, but any such inactivation must not have given rise to membrane-permeable degradation products. No information was available on the effect of ammonium chloride on lysosomal membrane permeability, therefore the mechanism of enhanced intracellular

inactivation could not be identified.

To summarise, uptake of $^{3}$H-leupeptin did not appear to be saturable but was energy dependent. The temperature dependence of uptake was marked but could not be used as a basis to distinguish between pinocytic and other modes of uptake. The rapid rate of release of radiolabel from $^{3}$H-leupeptin loaded yolk-sacs suggested that either degradation of leupeptin occurred, or that both the lysosomal and plasma membranes were more permeable towards leupeptin than towards pinocytic markers. The lack of effect of ammonium chloride indicates that the increased loss of leupeptin activity observed in the presence of ammonium chloride (Section 5.3.5) was not due to an increase in the amount released from the tissue (either intact or following degradation).

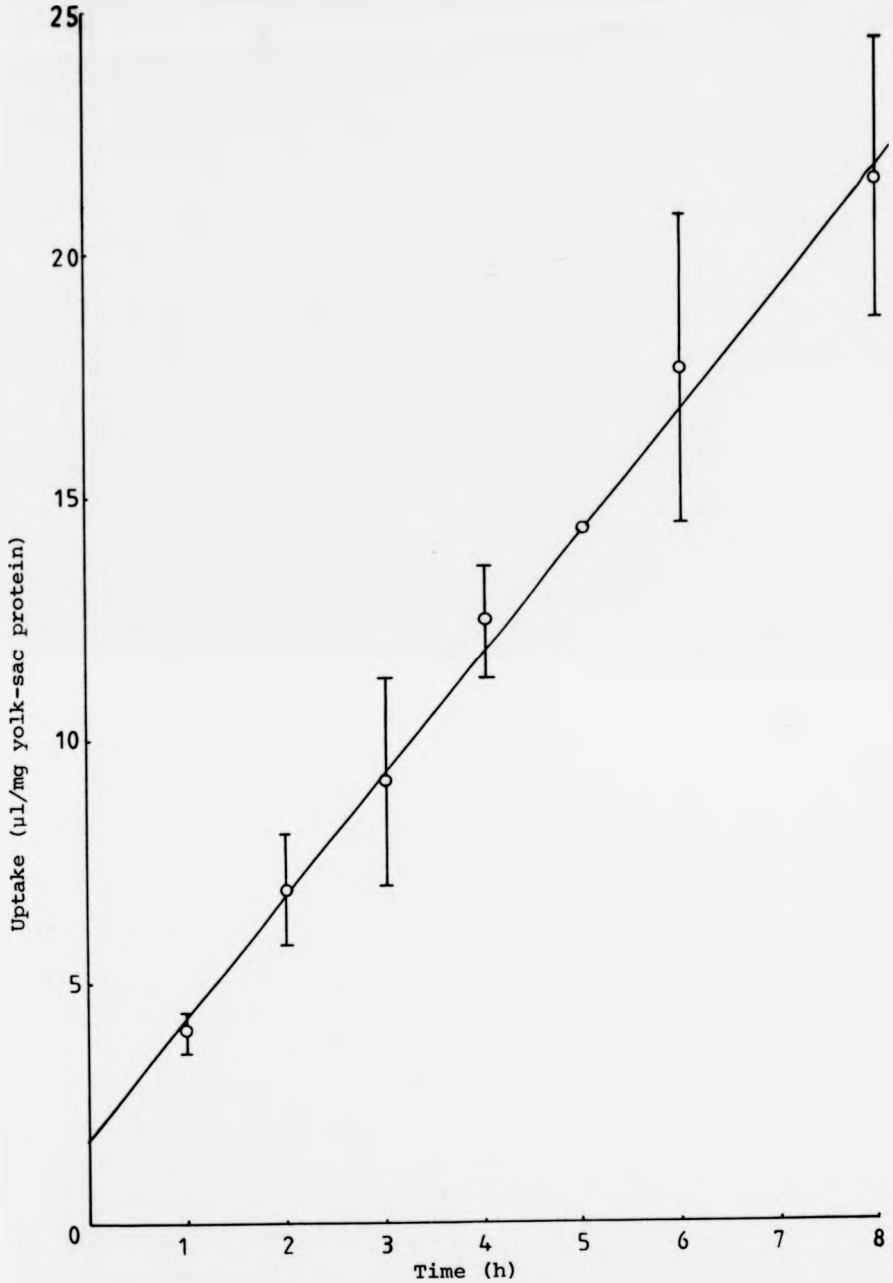



Fig. 6.1 Uptake of $^{3}$H-Inulin by Rat Yolk Sacs

Yolk sacs were incubated with $^{3}$H-inulin using the reduced volume method described in Section 2.2b. The amount of substrate within the tissue was calculated in terms of µl/mg yolk-sac protein as described in Section 2.7c. The graph shows the mean uptake values.

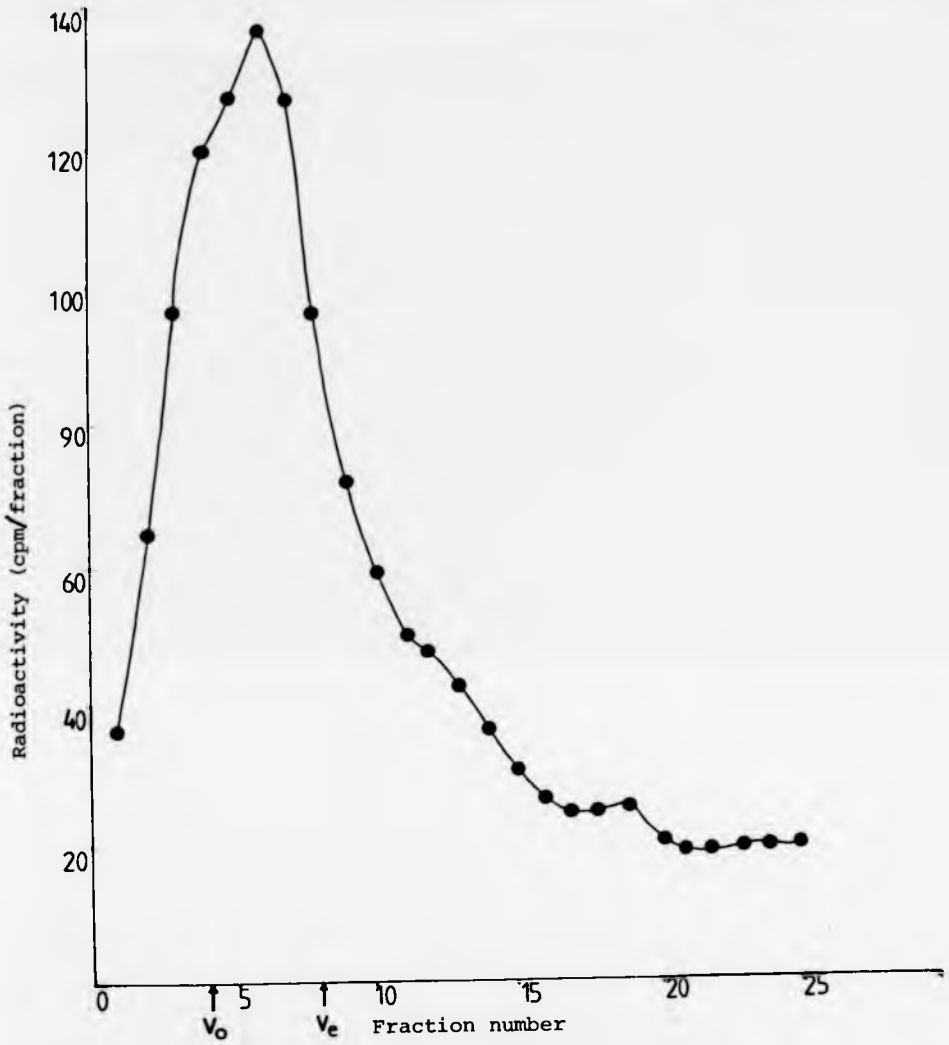



Fig. 6.2 Elution Profile of $^3$H-Radioactivity Released from Yolk Sacs Loaded with $^3$H-Inulin

Yolk sacs were incubated with $^3$H-inulin to load the tissue with substrate, then reincubated in fresh medium. Samples (1ml) of reincubation medium were loaded onto a small chromatography column containing Sephadex G25 (PD-10 column) and eluted with water. The eluant (collected in 1ml fractions) was assayed for radioactivity.

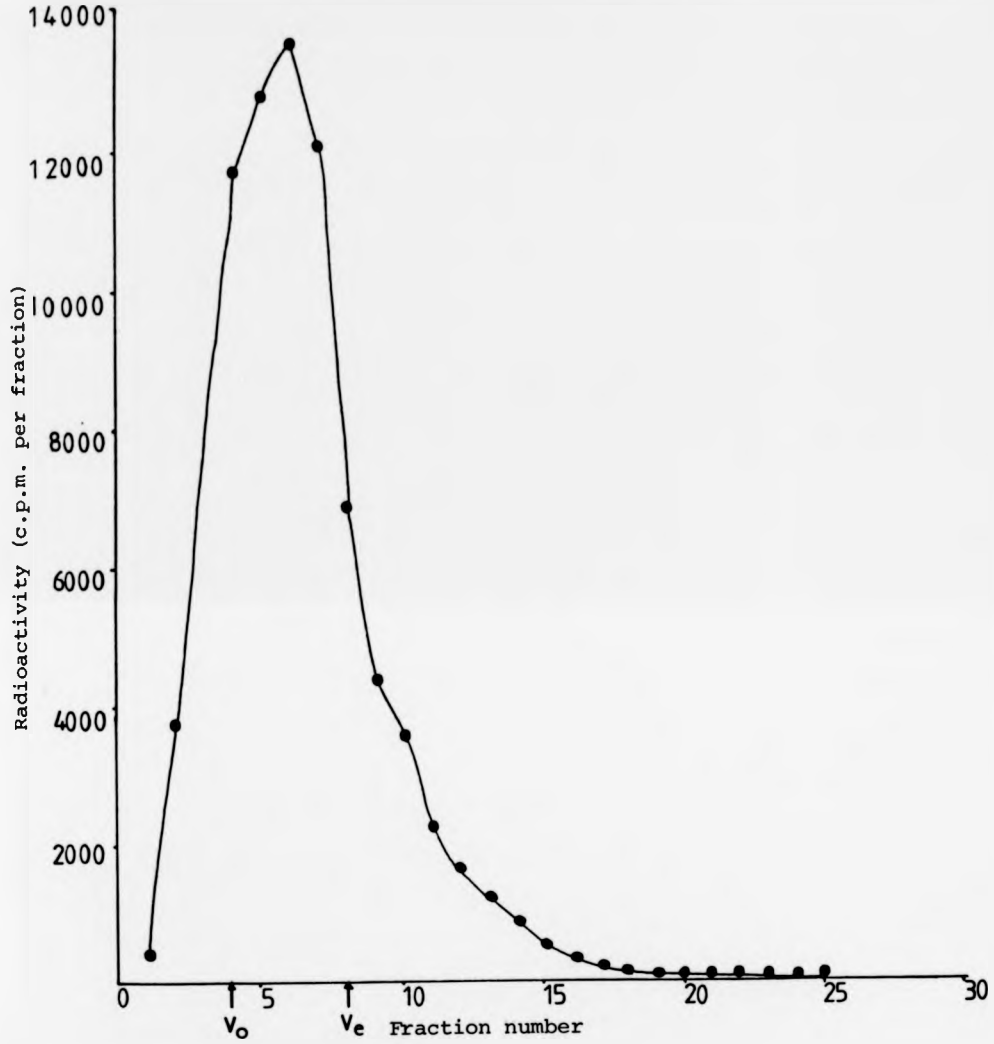



Fig. 6.3 Elution Profile of $^3$H-Inulin

$^3$H-inulin stock solution was diluted in distilled water. The diluted solution (1ml) was loaded onto a small chromatography column containing Sephadex G25 (PD-10 column) and eluted with water. The eluant (collected in 1ml fractions) was assayed for radioactivity.

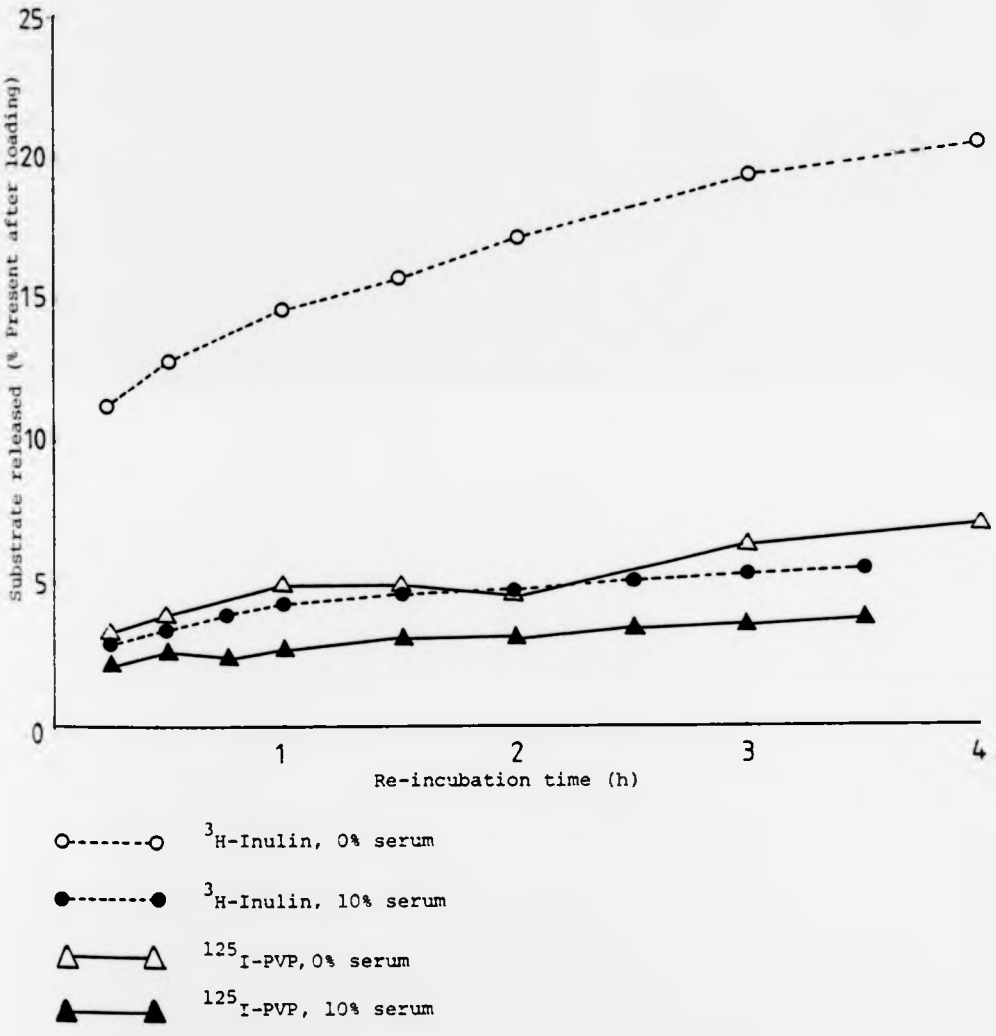
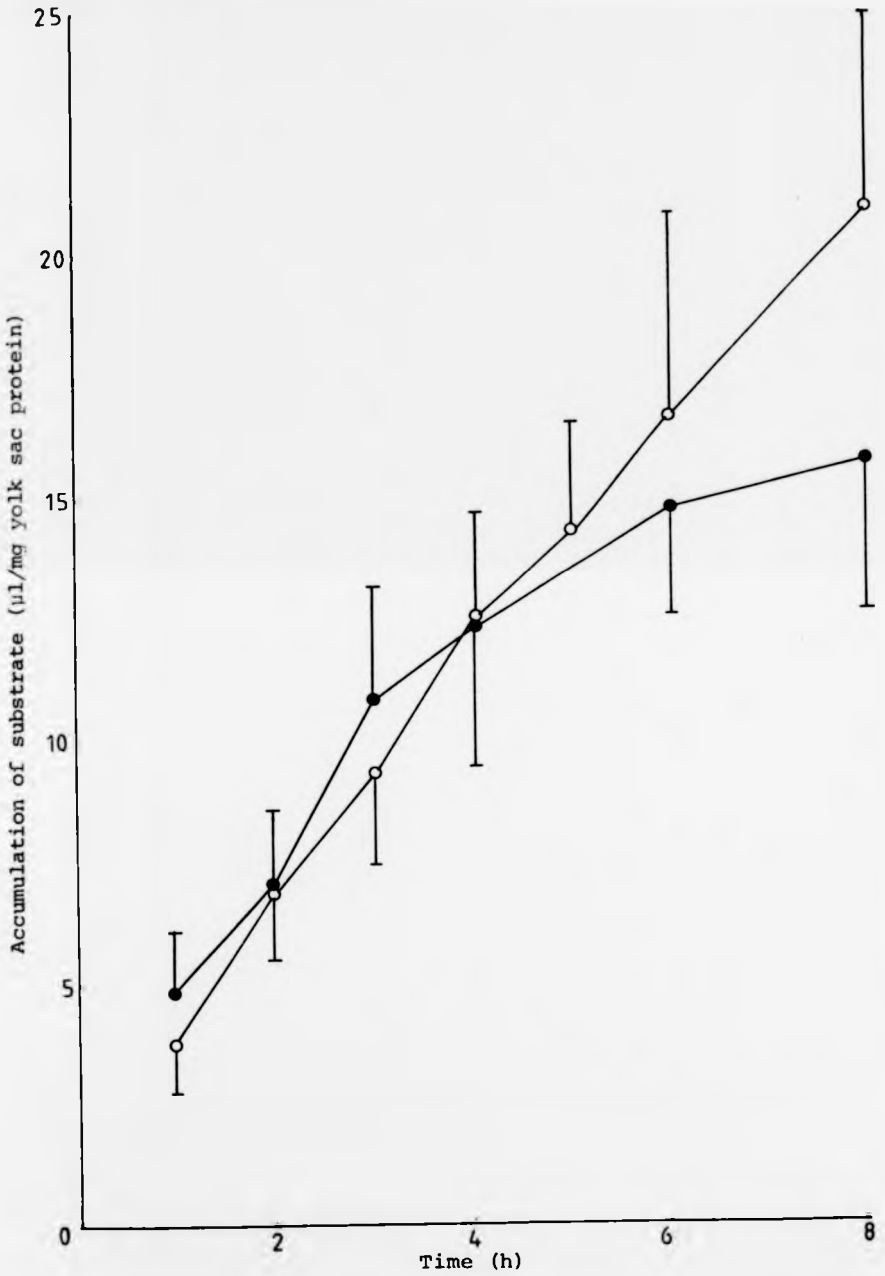



Fig. 6.4 Release of $^{125}$I-PVP and $^{3}$H-Inulin from Loaded Yolk Sacs in the Presence and Absence of Calf Serum

Yolk sacs were incubated in serum-free medium containing either $^{125}$I-PVP or $^{3}$H-inulin. The yolk sacs were then rinsed and re-incubated in substrate-free medium containing either no serum or 10% serum. Samples of re-incubation medium were removed for assay of radioactivity, as described in Section 6.2.2.




o $^{3}$H-inulin (various concentrations)

● $^{3}$H-leupeptin (various concentrations)

Fig. 6.5 Time-Course of Accumulation of $^{3}$H-Leupeptin Compared With Uptake of $^{3}$H-Inulin

Accumulation of substrate within yolk sacs was determined as detailed in Section 6.2.1b. Each point represents the mean accumulation (± S.D.), as described in Section 6.3.2.

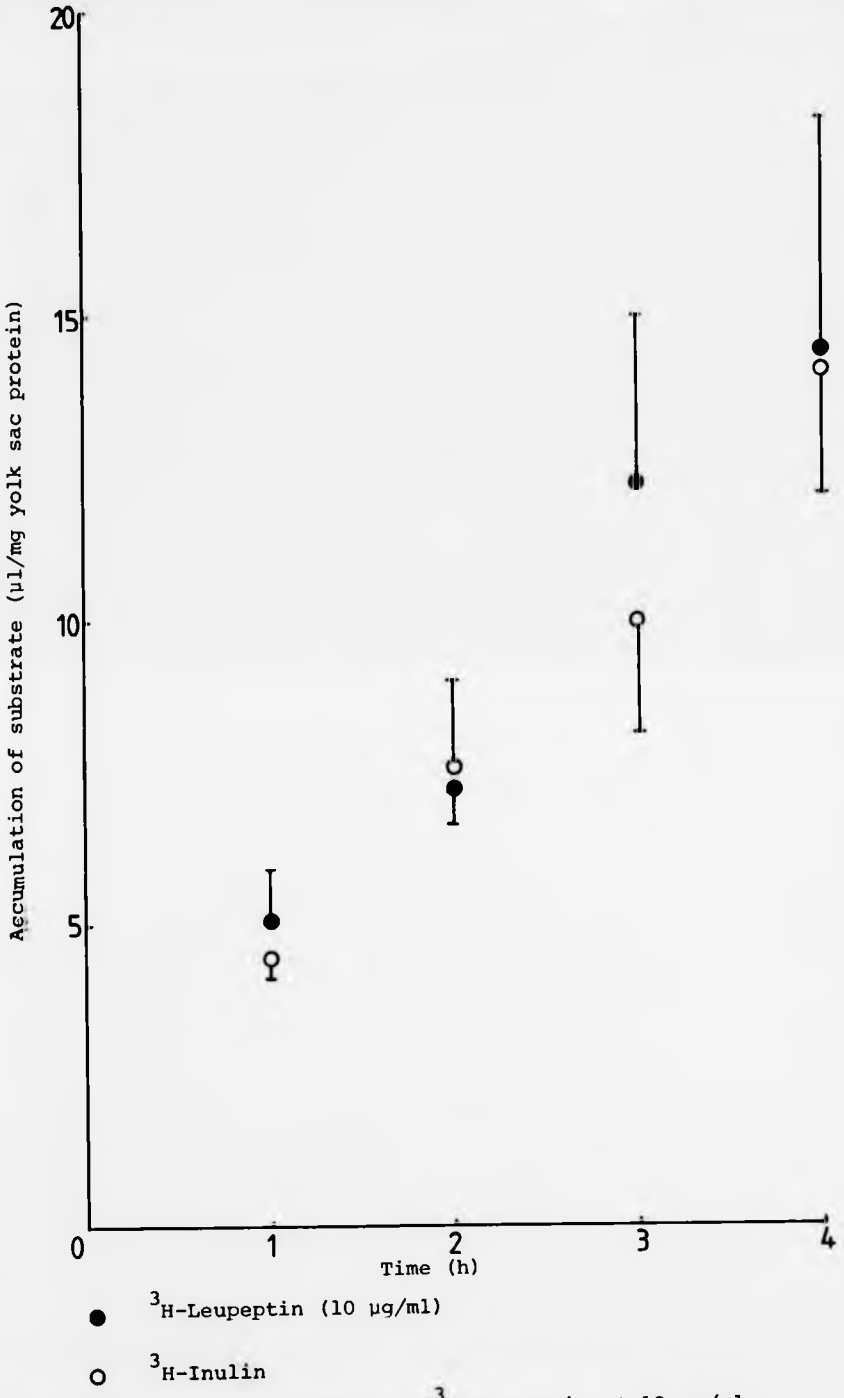



Fig. 6.6 Time-Course of Accumulation of $^{3}$H-Leupeptin at 10 µg/ml

Yolk sacs were incubated in medium containing $^{3}$H-leupeptin (10 µg/ml) and accumulation of radioactivity within the tissue measured as described in Section 6.2.1b. Uptake of $^{3}$H-inulin was determined in a matched control.

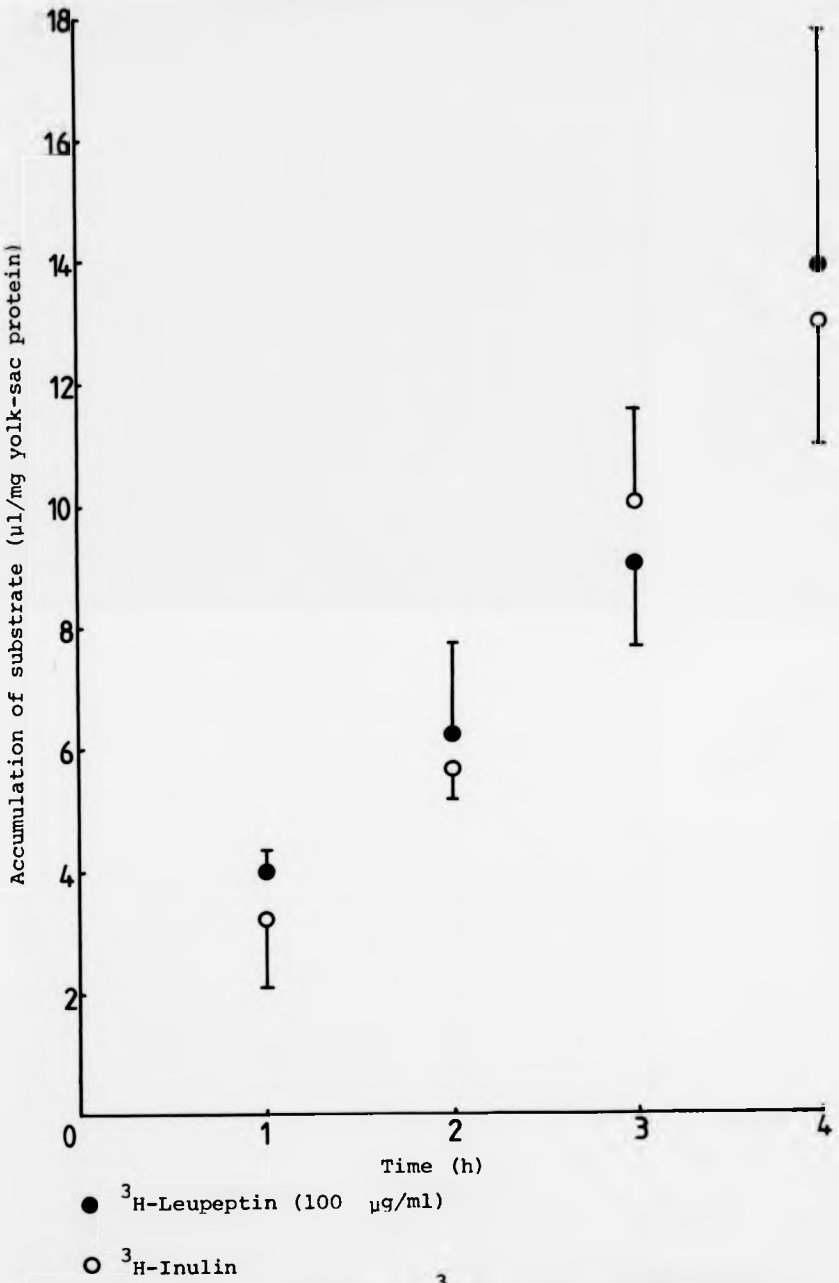



Fig. 6.7 Time Course of Accumulation of $^{3}$H-Leupeptin at 100 µg/ml

Yolk sacs were incubated in medium containing $^{3}$H-Leupeptin (10 µg/ml) and unlabelled leupeptin (90 µg/ml), and accumulation of radioactivity within the tissue measured as described in Section 6.2.1b. Uptake of $^{3}$H-inulin was determined in a matched control.

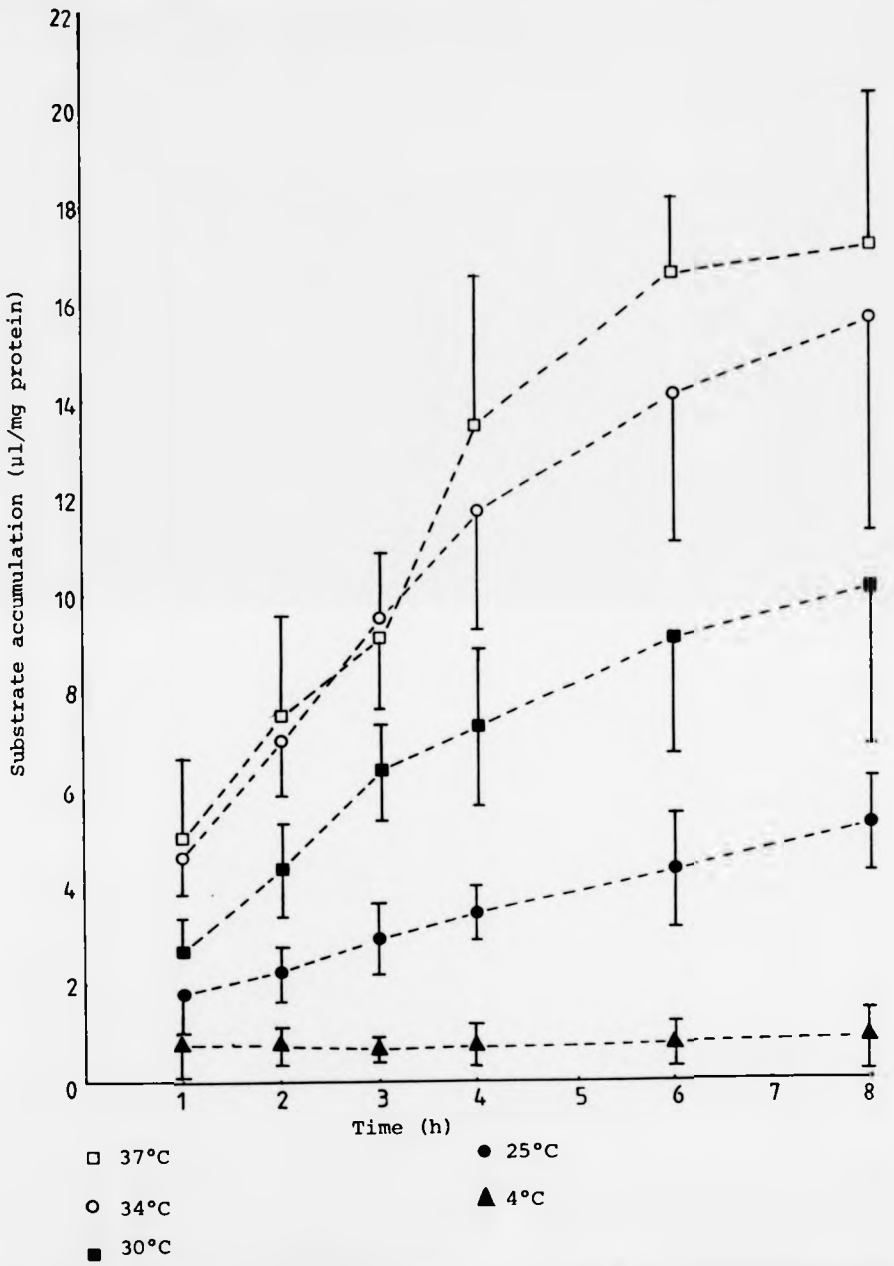



Fig. 6.8 Effect of Incubation Temperature on the Time-Course of Accumulation of $^{3}$H-leupeptin.

Yolk sacs were incubated in medium containing a trace of $^{3}$H-leupeptin (total leupeptin concentration 102.5 µg/ml) at the temperatures indicated. Accumulation of radioactivity was determined as detailed in Section 6.2.1b. Each point represents the mean accumulation (± S.D.) from at least 3 experiments.

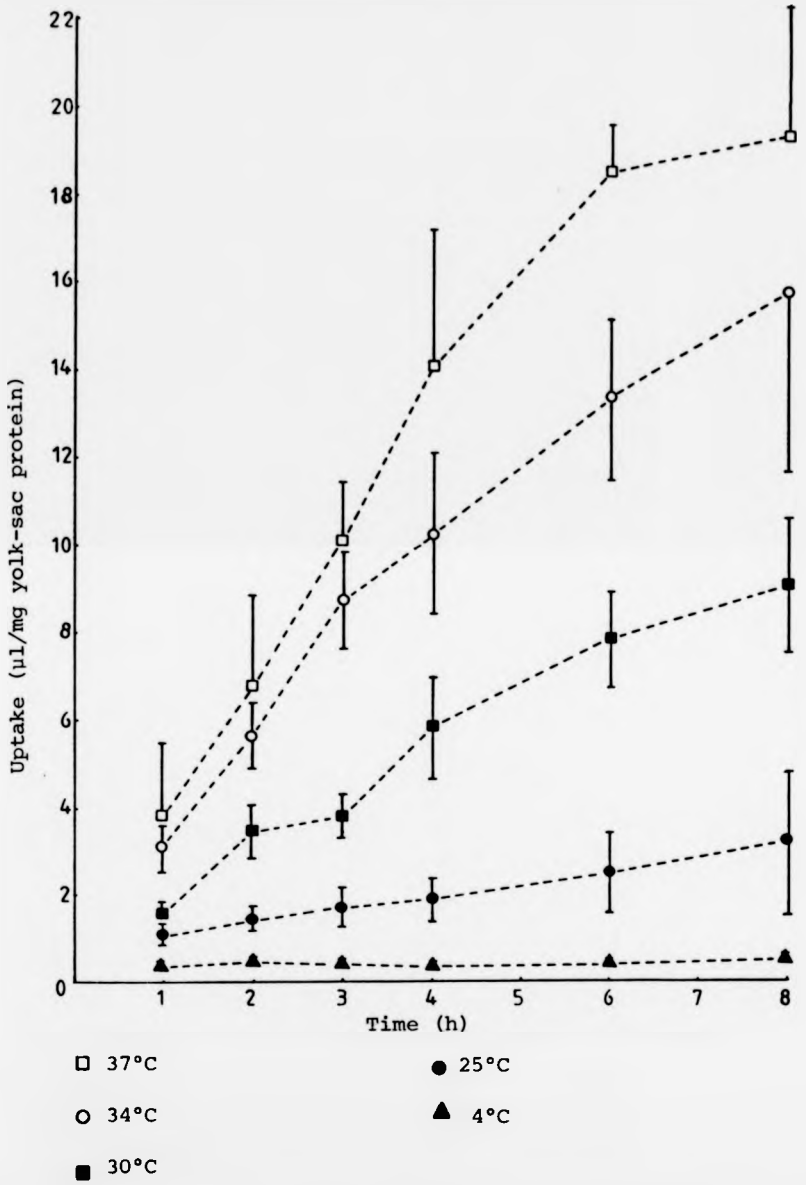



Fig. 6.9 Effect of Incubation Temperature on the Time-Course of Accumulation of $^{3}$H-Inulin

Uptake of $^{3}$H-inulin was monitored as matched controls to the experiments described in Fig. 6.8. Accumulation of $^{3}$H-inulin was determined as detailed in Section 6.2.1b. Each point represents the mean ( ±S.D.) from at least 3 experiments.

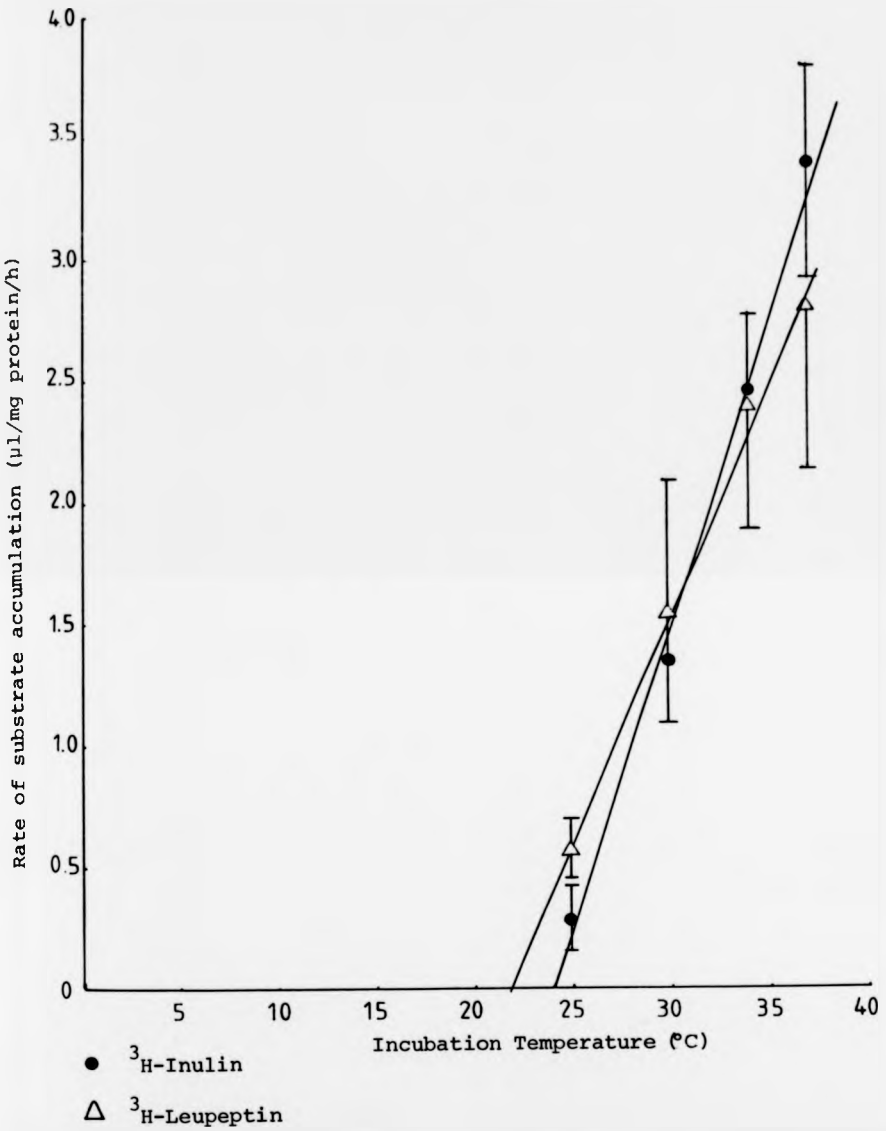



Fig. 6.10 Summary of the Effect of Temperature on the Rate of Accumulation of $^{3}$H-Leupeptin and $^{3}$H-Inulin

The rate of accumulation of substrate during the initial 4h incubation period was determined for each individual experiment described in Section 6.2.1. The mean rate of accumulation (± S.D.) was then plotted against the temperature of incubation.

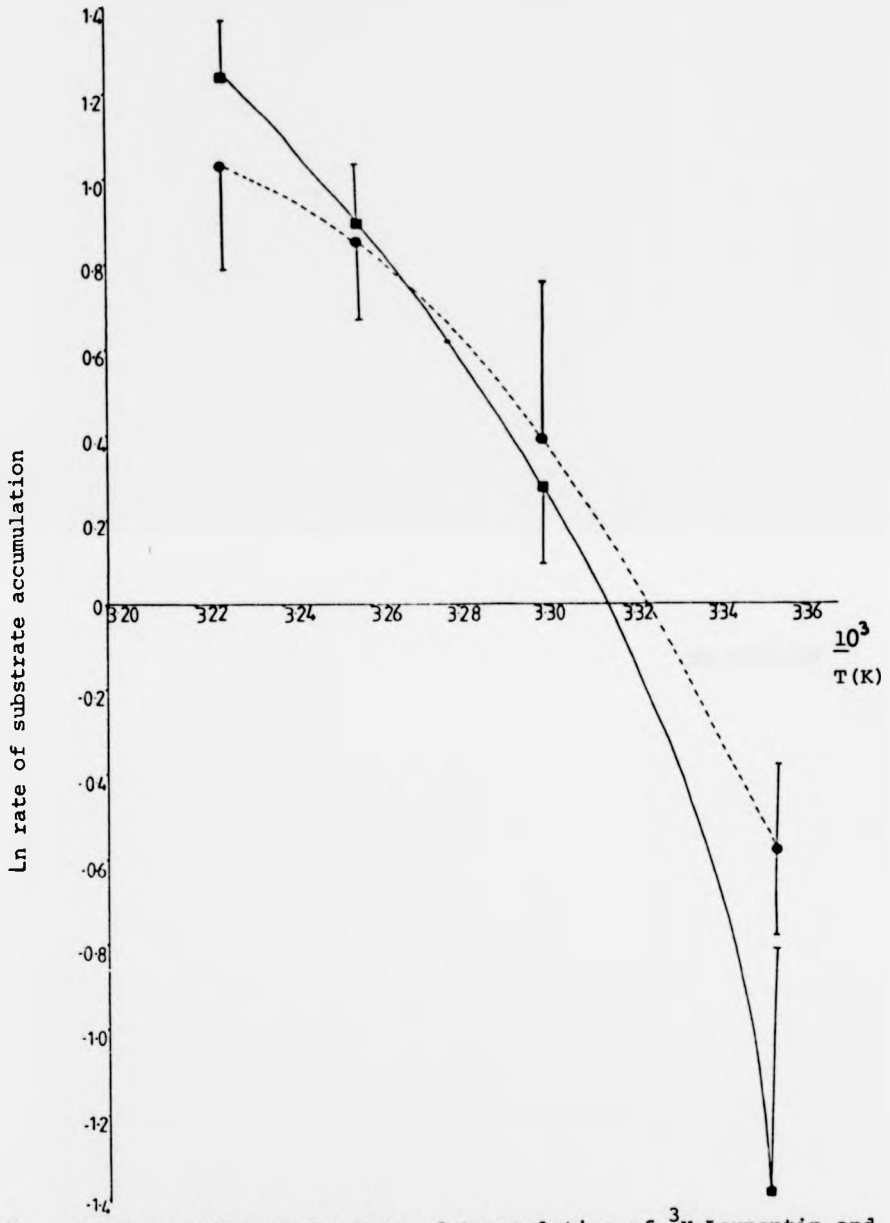



Fig. 6.11 Arrhenius Plot of the Rate of Accumulation of $^{3}$H-Leupeptin and $^{3}$H-Inulin

The natural log of the rates of accumulation of substrate determined for Fig. 6.10 were found, and the mean value (± S.D.) plotted against 1/T(K) for each substrate.

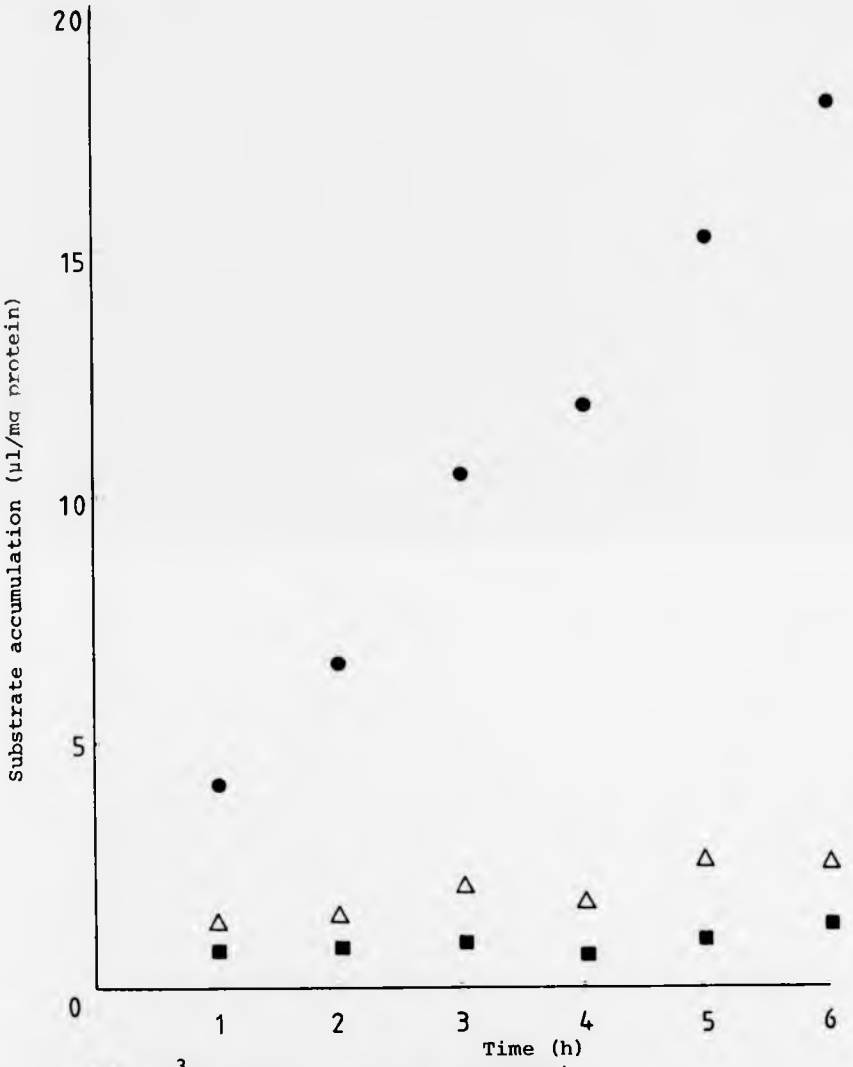



Fig. 6.12 Effect of Rotenone on the Accumulation of $^{3}$H-Leupeptin and $^{3}$H-Inulin

Yolk sacs were incubated with substrate in medium containing rotenone ($10^{-5}$M) as described in Section 6.2.1b. Uptake of $^{3}$H-inulin was also monitored in the absence of rotenone to indicate the rate of fluid-phase pinocytosis.

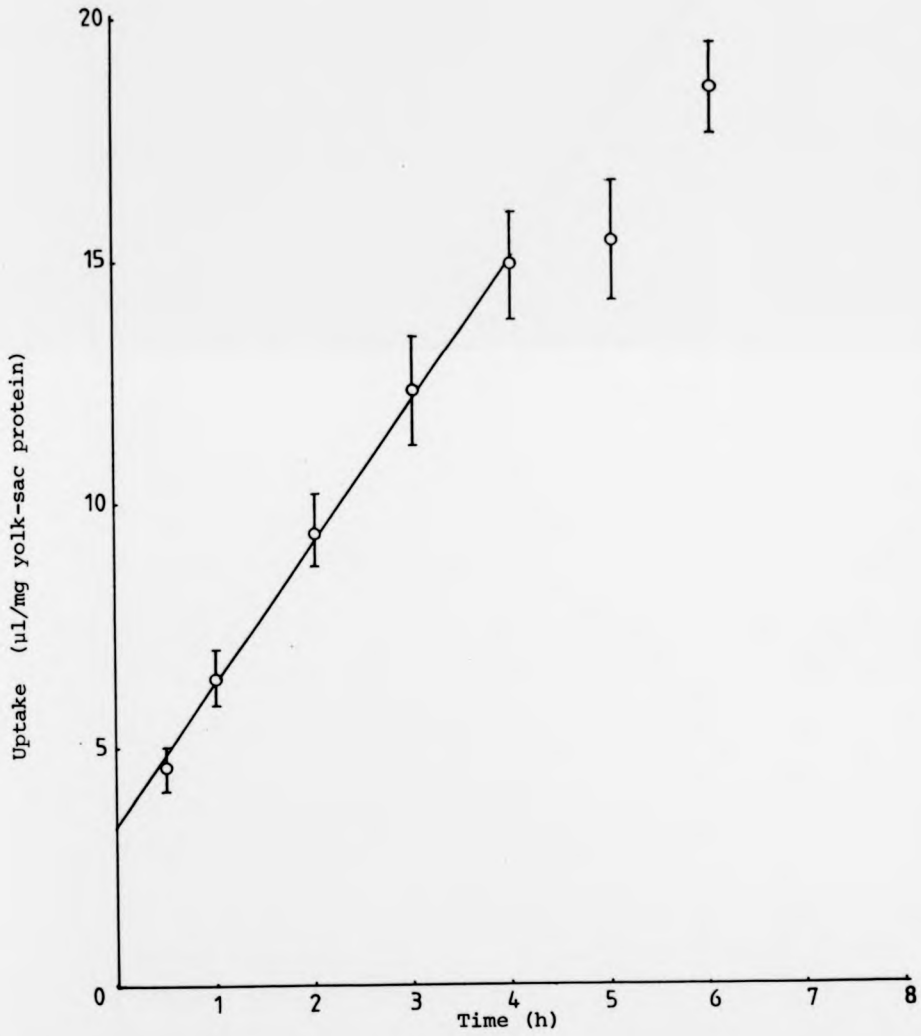



Fig. 6.13 Time-Course of Uptake of $^{14}$C-Sucrose

Uptake of $^{14}$C-sucrose was monitored as described for $^{3}$H-leupeptin in Section 6.2.1b. The mean uptake values (± S.D.) from 3 experiments are shown.

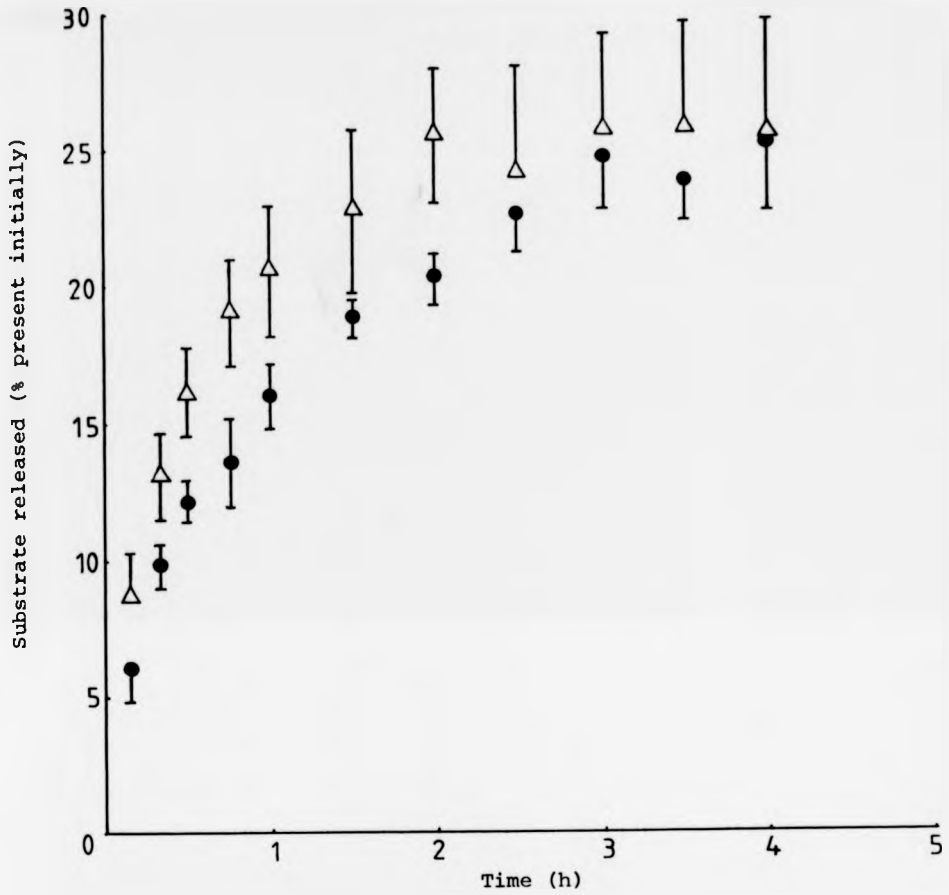
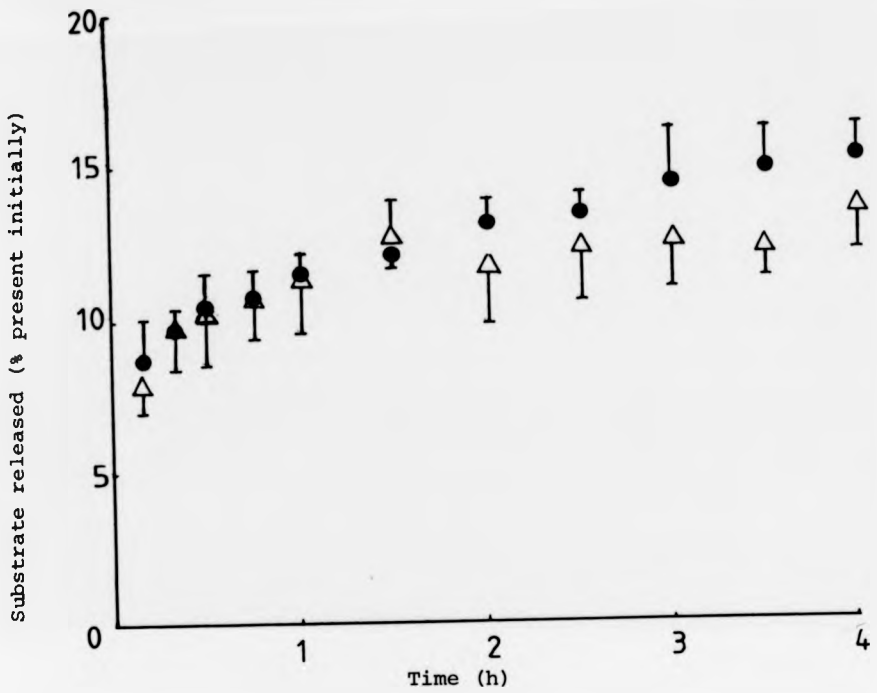



Fig. 6.14 Time-Course of Release of $^{14}$C-Sucrose from Loaded Yolk Sacs in the Presence and Absence of Ammonium Chloride

Tissue was incubated with $^{14}$C-sucrose then re-incubated in substrate-free medium in the presence and absence of ammonium chloride. The re-incubation medium was assayed for radioactivity as described in Section 6.2.2a,b. Results shown are the mean (± S.D.) from 3 experiments.




△ Ammonium chloride (10mM) present during re-incubation

● Ammonium chloride absent during re-incubation

Fig. 6.15 Time-Course of Release of $^{3}$H-Inulin from Loaded Yolk Sacs, in the Presence and Absence of Ammonium Chloride

Tissue was incubated as described in Section 6.2.2a and the results calculated as described in Section 6.2.2b.
Results shown are the mean (± S.D.) from 3 experiments.

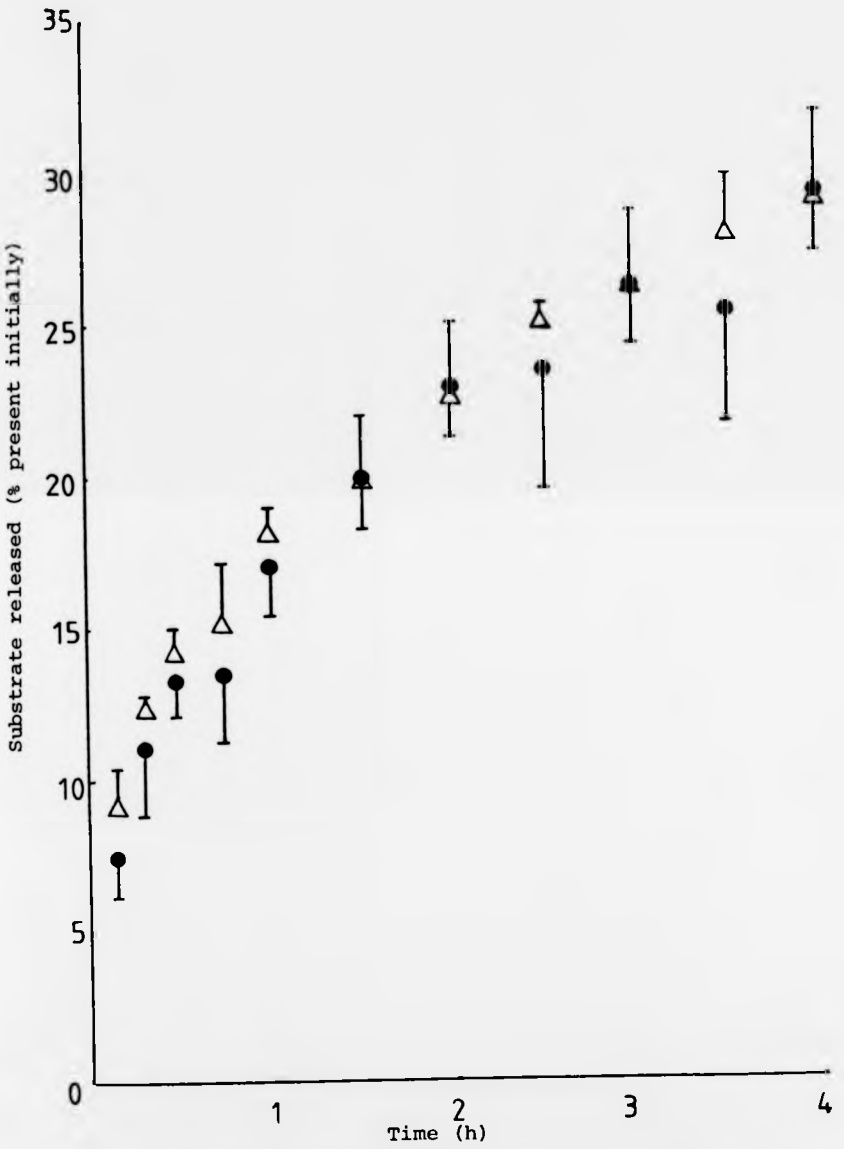



Fig. 6.16 Time-Course of Release of $^{3}$H-Leupeptin from Loaded Yolk Sacs, in the Presence and Absence of Ammonium Chloride

Tissue was incubated as described in Section 6.2.2a and the results calculated as described in Section 6.2.2b. Results shown are the mean (± S.D.) from 3 experiments.

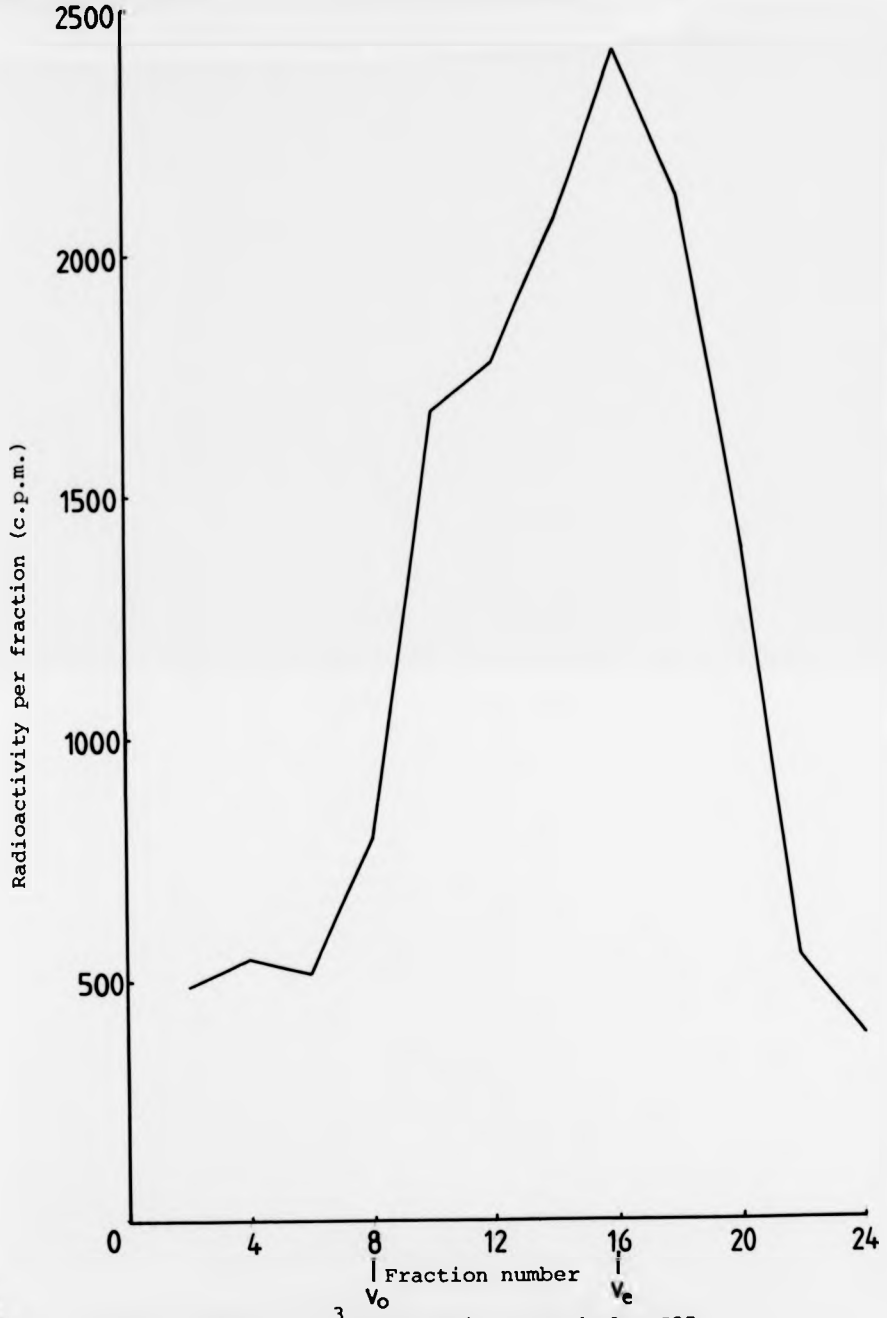



Fig. 6.17 Elution Profile of $^{3}$H-Leupeptin on Sephadex G25

A solution of $^{3}$H-leupeptin (0.5ml) was loaded onto a PD 10 column and eluted with water. The eluant (0.5ml fractions) was assayed for radioactivity, as described in Section 6.2.3.

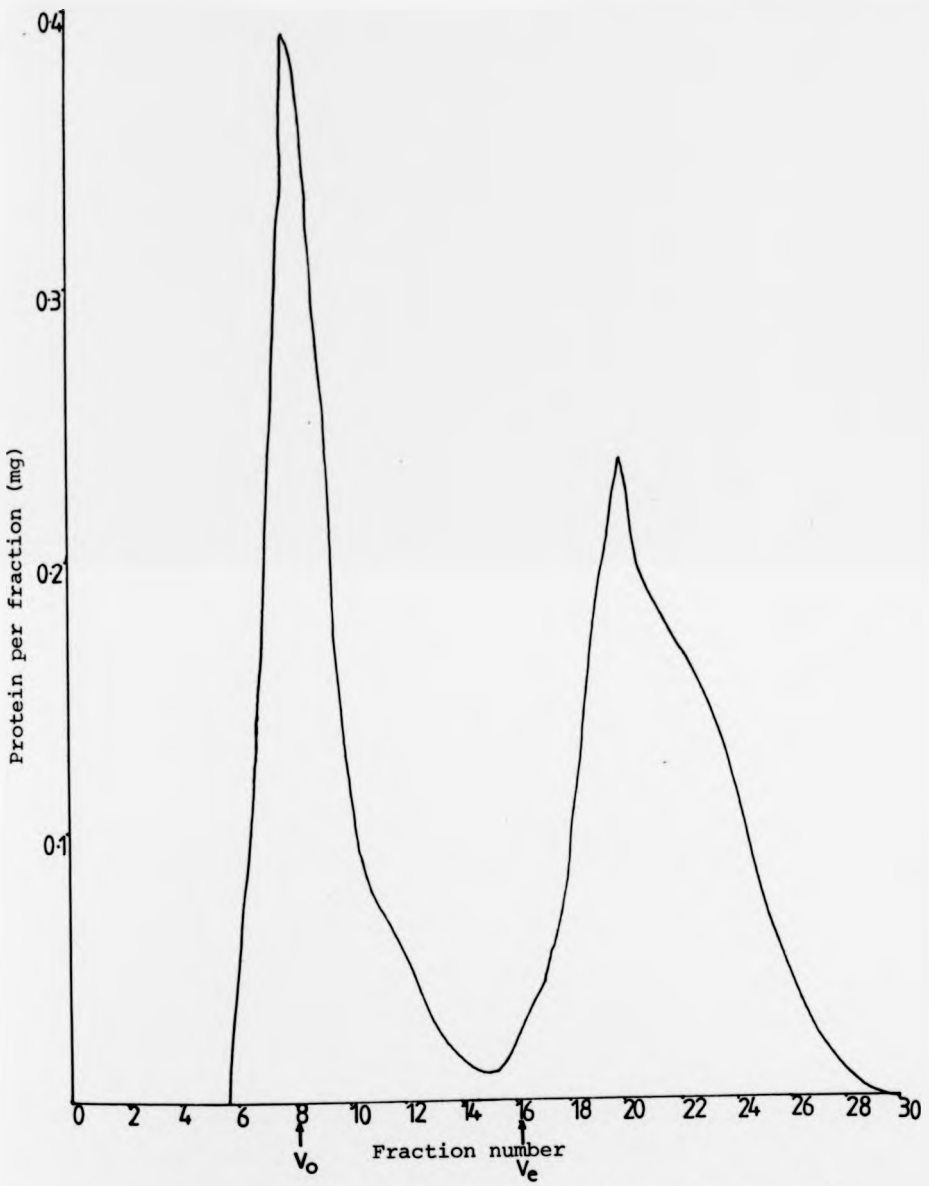



Fig. 6.18 Elution Profile of Yolk-Sac Proteins on Sephadex G25

A cell-free yolk-sac homogenate (0.5ml) was loaded onto a PD-10 column and eluted with water. The eluant (0.5ml fractions) was assayed for protein by the Folin assay as described in Section 6.2.3.

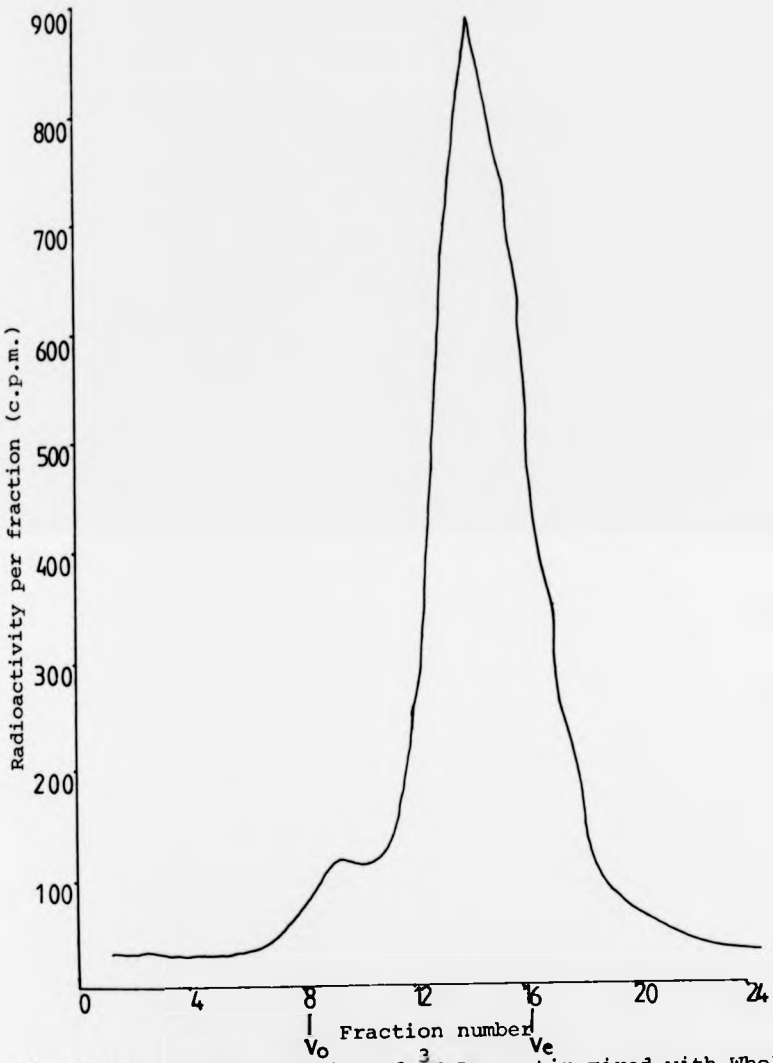



Fig. 6.19 Elution Profile of $^{3}$H-Leupeptin mixed with Whole Yolk-Sac Homogenate on Sephadex G25

A solution of $^{3}$H-leupeptin was added to a whole yolk-sac homogenate then the mixture centrifuged to remove tissue debris. The supernatant (0.5ml) was loaded onto a PD-10 column and eluted with water. Fractions (0.5ml) of the eluant were assayed for radioactivity, as described in Section 6.2.3.

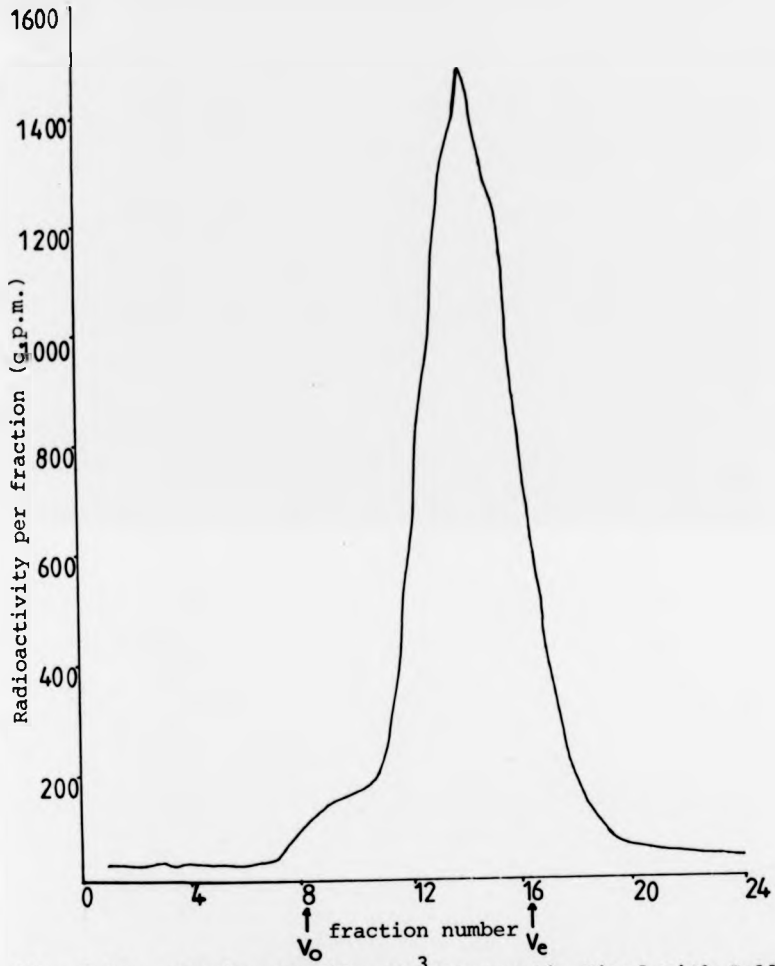



Fig. 6.20 Elution Profile of $^{3}$H-Leupeptin Mixed with Cell-Free Yolk-Sac Homogenate

A solution of $^{3}$H-leupeptin was added to the supernatant of a yolk-sac homogenate that had been centrifuged to remove tissue debris. The mixture (0.5ml) was loaded onto a PD-10 column and eluted with water. Fractions (0.5ml) of the eluant were assayed for radioactivity as described in Section 6.2.3.

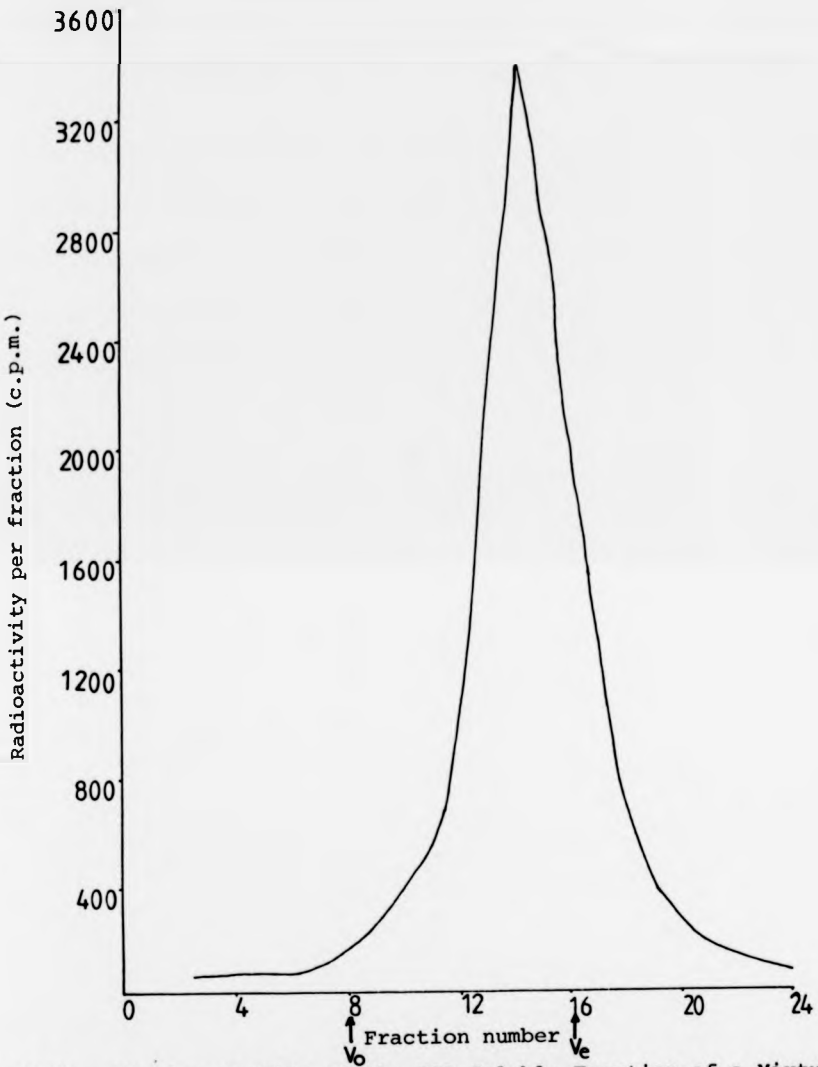



Fig. 6.21 Elution Profile of the TCA-Soluble Fraction of a Mixture of $^{3}$H-Leupeptin and Yolk-Sac Homogenate

$^{3}$H-leupeptin was added to a whole yolk-sac homogenate then the yolk-sac proteins precipiated with TCA. A sample (0.5ml) of the TCA soluble fraction was loaded onto a PD-10 column and eluted with water. Fractions (0.5ml) of the eluant were assayed for radioactivity, as described in Section 6.2.3.

Table 6.1

Effect of Concentration on the Rate of Accumulation of Leupeptin

Yolk sacs were incubated with $^{3}$H-Leupeptin (or a mixture of $^{3}$H-leupeptin and unlabelled leupeptin) as described in Section 6.2.1b. The rate of accumulation was calculated from the mean value of accumulation at each time point for at least 2 experiments. The rate of uptake of $^{3}$H-inulin was determined in the same way as matched controls.

| Leupeptin Conc. | $^{3}$H-Leupeptin Rate of Accumulation (µl/h/mg protein) | Correlation Coefficient | $^{3}$H-Inulin Rate of Uptake (µl/h/mg protein) | Correlation Coefficient |
|---|---|---|---|---|
| 3 | 2.58 | 0.97 | 2.50 | 0.98 |
| 5 | 2.45 | 0.99 | 2.49 | 0.98 |
| 10 | 3.17 | 0.99 | 3.57 | 0.99 |
| 50 | 2.98 | 0.97 | 3.63 | 0.99 |
| 100 | 2.71 | 0.96 | 3.87 | 0.99 |
| Mean Rate of Accumulation over concentration range (±S.D.) | 2.78 (±0.29) | | 3.39 (±0.61) | |

# CHAPTER 7

# EFFECT OF LEUPEPTIN ON PROTEIN DEGRADATION WITHIN INTACT LYSOSOMES

## 7.1 INTRODUCTION

Although many reports indicate that leupeptin is able to inhibit lysosomal enzymes *in situ* (eg Knowles *et al.*, 1981; Seglen *et al.*, 1979), thus must be able to enter lysosomes, the possibility of leupeptin acting at other intracellular sites cannot be eliminated since it is capable of inhibiting both lysosomal and non-lysosomal proteinases *in vitro*. However its effects *in vivo* may be limited by restriction of its access to potential sites of action. Evidence for inhibition by leupeptin of non-lysosomal proteolytic enzymes in intact cells is lacking, though most studies do not eliminate the possibility of such action (eg Libby & Goldberg, 1978; Cockle & Dean, 1982). Indeed Seglen *et al.* (1979) & Grinde and Seglen (1980) found that leupeptin-induced inhibition of proteolysis in hepatocytes was slightly additive to that of weak bases, and concluded that the additional effect was caused by inhibition of a non-lysosomal pathway of protein degradation.

There are two main mechanisms by which leupeptin could enter cells: direct plasma-membrane permeation or pinocytosis. If the former is the main route of entry, leupeptin will initially be located in the cytosol. In order to affect lysosomal enzymes, permeation across the lysosomal membrane must then follow. If uptake of leupeptin occurs via pinocytosis, the leupeptin will initially be located in the vacuolar system, hence permeation across the lysosomal membrane will not be required for inhibition of lysosomal enzymes. However the effects of the inhibitor may not necessarily be confined to the lysosomes if permeation of the intact molecule across the lysosomal membrane occurs.

The work described in this chapter attempts to determine whether leupeptin is able to permeate lysosomal membranes and so has access

to both lysosomal and non-lysosomal compartments, whatever its mode of uptake. The experiments were performed using rat-liver lysosomes rather than lysosomes isolated from yolk-sac tissue. This is because, although exhaustive attempts have been made in the past to isolate intact yolk-sac lysosomes, the majority appeared to rupture during homogenization (Williams & Lloyd, unpublished observations). Even if isolation of intact lysosomes from yolk sacs were possible the isolation of a sufficient quantity of protein-loaded lysosomes for *in vitro* studies would prove difficult.

Several different methods have been utilized to demonstrate the permeability properties of lysosomes. Traditionally, either the ability of an agent to afford osmotic protection to isolated lysosomes (eg see Lloyd 1969; 1971), or an assessment of the degree of vacuolization of lysosomes within intact cells caused by the agent (eg Ehrenreich & Cohn, 1969) have been used. Briefly, the rationale behind the osmotic protection method is that when lysosomes are suspended in an isotonic solution of a permeant molecule, entry of this molecule into lysosomes (along the concentration gradient) will lead to an osmotic imbalance. Influx of water then causes lysosomal expansion and eventual rupture of the membrane. This lysis can be detected by measuring the percentage of free, as opposed to latent, lysosomal enzyme activity. The vacuolization method is based on the fact that when non-permeant molecules enter lysosomes (following pinocytic uptake into cells), an osmotic inbalance is created and water enters the lysosomes causing swelling. Hence, if vacuolization is observed on incubating cells with an agent, that agent must be incapable of permeation through the lysosomal membrane.

Both these methods have attendant disadvantages, some of which are discussed by Reijngound & Tager (1977), and by Hales *et al.* (1984). The osmotic protection method requires careful controls to

ensure that the substrate used to measure free enzyme activity cannot permeate the lysosomal membrane, or that, if centrifugation is employed to separate 'free' from 'latent' enzyme activity, the enzyme being assayed does not adsorb to lysed lysosomal membranes. The method also has limitations in terms of the solubility, cost, and the non-physiological concentration of the agent used. For the vacuolization method, only substrates that are taken up by pinocytosis and which are not digested by lysosomal enzymes can be employed. (This may prevent peptides from being used as vacuolating agents.) Quantitation is difficult, and generally only cells or tissues that can be conveniently visualized by light microscopy may be used.

Some of the conclusions drawn by the early workers using these methods are now under review. In the past, movement across the lysosomal membrane has been attribted to passive diffusion. The rate of permeation of an agent has been correlated with its size, hydrophobicity and charge (eg Lloyd, 1973; Reingoud & Tager, 1977). More recent evidence suggests that movement of at least some molecules is by specific transport systems (Hales et al., 1984).

Permeation of several molecules across the lysosomal membrane has been monitored using specially-designed techniques. For example, cystine transport has been investigated in great detail, in an attempt to discover the primary defect in the disease cystinosis. Lysosomes were loaded with cystine then the release of this molecule monitored. Transport was found to be pH and ATP dependent, and did not occur normally in cystinotic cells (for review, see Schneider et al., 1984). This suggests the presence of a specific, energy-dependent transport system for cystine in the lysosomal membrane.

Pepstatin, which is a microbial proteinase inhibitor containing

five amino acid residues, was investigated by introducing it into rat liver lysosomes *in vivo*, as an asialofetuin-pepstatin complex. After intravenous injection of the complex, pepstatin-loaded lysosomes were isolated from the liver and incubated *in vitro*. The time-course of release of pepstatin was found to be linear, and 60% of the inhibitor initially present was released over 60 minutes (Furuno *et al.*, 1983).

Lysosomal membrane permeation by agents that affect protein degradation can be detected by monitoring their effect on the rate of intralysosomal proteolysis in isolated lysosomes incubated *in vitro*. A method frequently used is based on one developed by Mego & McQueen (1965 a,b). Liver lysosomes are loaded *in vivo* with a radiolabelled protein (usually $^{125}I\text{-}BSA_{fd}$) by means of intravenous injection of the protein. The protein is then cleared from the bloodstream, mainly by the liver, via pinocytosis. The liver lysosomes are then isolated and incubated in an isotonic solution in the presence and absence of the proteolytic effector. The labelled protein is degraded within the lysosomes and the time-courses of release of low-molecular-weight degradation products monitored and compared (Mego & McQueen, 1965 a,b; Mego *et al.*, 1967; Davies *et al.*, 1971; Reijngoud *et al.*, 1976). [When using this method it is important to establish that the observed proteolysis occurs only within the lysosomes. Many factors are known to affect lysosomal integrity *in vitro*, therefore ideally, optimum conditions of pH, temperature, medium composition etc. to maximize lysosomal membrane stability should be used, and lysosomal rupture monitored.] The effects of proteolytic inhibitors such as ammonia, methylamine (Reijngoud *et al.*, 1976), iodoacetate (Mego & McQueen, 1965a), and stimulators such as glutathione (Mego, 1984) and cysteine (Mego & McQueen, 1965a) have been investigated using this system. (The results of these investigations will be discussed in Section 7.4).

The effect of leupeptin on protein degradation within lysosomes has also been investigated. Neff et al. (1979) added leupeptin to a suspension of lysosomes (that did not contain any radioabelled protein), and measured the rate of production of fluorescamine-positive material arising from degradation of the heterogeneous mixture of proteins present within lysosomes. Leupeptin did not affect the rate of degradation unless the membranes were first lysed by freeze/thaw action, suggesting that leupeptin was not able to permeate through membranes. However, Ahlberg & Glaumann (1985) used leupeptin successfully to inhibit degradation of proteins taken up into lysosomes by microautophagy. The lysosomal membrane was assummed to be fully permeable to leupeptin in that study.

For the experiments described in this chapter, lysosomes were loaded with $^{125}I\text{-}BSA_{fd}$ using the method of Mego & McQueen (1965a), then incubated in the presence of leupeptin. If the rate of release of TCA-solubles was lowered compared with controls, leupeptin must either be able to enter the lysosomes, or cause lysis of isolated lysosomes. These results were compared with the effects of ammonium chloride, which is reported to permeate lysosomal membranes (Ohkuma & Poole, 1981; Poole & Ohkuma, 1981; Reijngoud et al., 1976).

The medium composition, [eg presence or absence of ATP and $Mg^{2+}$ (Mego & McQueen, 1965a,b; Ohkuma et al.,1982), ionic strength (Mego et al., 1967), molarity (Mego et al., 1967; Lloyd, 1973), pH and type of buffer (Mego, 1971; Mego et al., 1972)] and temperature of incubation (Davis et al., 1971) are all known to affect the rate of intralysosomal proteolysis and/or the degree of lysis of the lysosomes. Some of these factors were investigated in the work reported in this chapter.

In summary, the following were investigated in this chapter:-

i) Rat-liver lysosomes loaded with $^{125}$I-BSA$_{fd}$ were incubated either in sucrose (0.25M), or in medium containing sucrose (0.2mM), KCl (50mM), $MgCl_2$(10mM), ATP (1mM), and HEPES (20mM), buffered at pH 7.0. The rate of production of TCA-solubles was monitored to determine the effect of the more complex ATP-medium on the rate of degradation.

ii) The effects of Triton X-100 (0.1%) on the rate of degradation of $^{125}$I-BSA$_{fd}$ was determined, to assess whether proteolysis could occur once the lysosomal contents had been released into the medium after lysis of the membrane by the detergent.

iii) The rate of production of TCA-soluble material from $^{125}$I-BSA$_{fd}$-loaded lysosomes was monitored in the presence of ammonium chloride (20mM) or leupeptin (10µg/ml) to establish whether either inhibitor was able to pass into the lysosomes and inhibit degradation.

iv) Leupeptin was added to a suspension of $^{125}$I-BSA$_{fd}$-loaded lysosomes in sucrose (0.25M) to give a final leupeptin concentration of 10, 25 or 100µg/ml. The rates of production of TCA-soluble material were then monitored, to determine the degree of inhibition at each concentration.

v) The pH of the lysosomal suspension was varied, using a medium composed of sucrose (0.25M) and phosphate buffer (30mM). The rate of production of TCA-soluble material was thus measured at pH 6, 7 and 8. The effects of ammonium chloride (20mM) and leupeptin(10µg/ml) were determined at each pH.

vi) An estimate of the amount of lysosomal lysis that occurred during each of the above experiments was made by measuring the amount of intact $^{125}$I-BSA$_{fd}$ that became released into the incubation medium.

## 7.2. MATERIALS AND METHODS

### I. MATERIALS

| | |
|---|---|
| Rats | Adult male Wistar, weight approximately 250g. Rats were starved overnight before use to deplete stored glycogen from the liver. |

**Equipment**

| | |
|---|---|
| Centrifuge | MSE High Speed 18. M.S.E., London, U.K. |

All other equipment as described in Chapter 2. Glassware and polypropylene vessels were soaked in acid (dilute HCl) before use to remove any traces of detergent.

**Reagents**

| | |
|---|---|
| $^{125}I\text{-}BSA_{fd}$ | Prepared as described in Section 2.1a. Used as undiluted, dialysed solution. |
| Leupeptin | Portions of a deep frozen stock solution (2mg/ml in water) were diluted to final concentrations of 10, 25 and 100µg/ml with the lysosomal suspension. |
| Ammonium Chloride | A stock solution (0.2M) in sucrose (0.25M) was diluted to a final concentration of 20mM with the lysosomal suspension. |
| Triton X-100 | A stock solution (10% v/v) was diluted to 0.1% with the lysosomal suspension. |

Suspension Media:

| | |
|---|---|
| Sucrose | 0.25M, unbuffered. |
| Buffered sucrose | 0.5M sucrose mixed with an equal volume of $KH_2/Na_2H$ phosphate buffer (60mM) of the appropriate pH. |
| ATP medium | Solutions of HEPES buffer (pH 7.0, 0.2M, 5ml) KCl(0.25M, 10ml), $MgCl_2$ (0.5M, 2ml) and sucrose (0.5M, 20ml), were mixed together and the total volume adjusted to 50ml. Just before use a freshly prepared solution of ATP (100mM, 10µl/ml medium) was added, and the pH readjusted to 7 if necessary. (This resuspension medium is similar to that used by Ohkuma et al., 1982.) |

All other reagents as described in Chapter 2.

## II. METHODS

### 7.2.1. Preparation of $^{125}$I-BSA$_{fd}$-loaded lysosomes

The method used was based on that described by Mego & McQueen (1965 a,b). Rats were anaesthetized with ether to allow an intravenous injection of $^{125}$I-BSA$_{fd}$ (approximately 1ml per animal) into the femoral vein. After exactly 30min, during which period the $^{125}$I-BSA$_{fd}$ was cleared from the bloodstream and reached a maximum concentration in liver lysosomes (Mego & McQueen 1965a), the animal was sacrificed. The liver was flushed with ice-cold sucrose (0.25M, about 50ml, introduced via the vena cava), to remove blood from and to rapidly cool the organ.

Preparation of the lysosomal-rich fraction was based on the method of DeDuve et al.(1955). The liver was removed from the animal, chopped roughly with scissors and passed through a sieve. The resulting pulp was weighed and sucrose solution (0.25M) added to give a suspension approximately 40% (w/v). This was homogenized using a Teflon-on-glass Potter-Elvehjem-type homogenizer (3-5 passes of the pestle over 30secs) and diluted with additional sucrose solution to give a 10% w/v suspension. The homogenate was then centrifuged at 1100g for 10 min to remove nuclei and unbroken cells, and the supernatant carefully decanted into fresh centrifuge tubes. This was then centrifuged at 22,500g for 10 minutes to obtain a fraction (pellet) enriched in lysosomes. (The fraction would also contain a large proportion of mitochondria.) After removing the supernatant, the pellets were gently resuspended in approximately 100ml of the suspension medium (ie. sucrose, buffered sucrose, or ATP medium). All material was kept cool (either on ice or in a cooled centrifuge), and preparation carried out as quickly as possible, to minimise degradation of $^{125}$I-BSA$_{fd}$.

7.2.2. Measurement of Rate of Degradation of $^{125}$I-BSA$_{fd}$ by Lysosomes

The lysosomal suspension was measured into 25ml plastic, sealable universals, which were incubated at 25°C. The test agent (leupeptin, ammonium chloride or Triton X-100) was added at zero time and duplicate samples (1ml) of the suspension were removed at t = 0, 15, 30, 60, 90, 120 and 150 minutes, to assess the amount of TCA-soluble radioactive material produced, and hence monitor the time-course of $^{125}$I-BSA$_{fd}$ degradation. On removal, the samples were added to vials containing aqueous TCA solution (20% w/v, 0.5ml) which precipitated undegraded $^{125}$I-BSA$_{fd}$ and intact proteins. (TCA has been shown to release the contents of lysosomes; Mego & McQueen, 1965a,b; thus it was not necessary to disrupt the lysosomal membrane before precipitating the $^{125}$I-BSA$_{fd}$.) The total radioactivity of the samples was determined and the TCA-precipitate pelleted by centrifugation at 1000g for 20min. The TCA-soluble supernatant was then decanted into fresh vials and assayed for radioactivity. The percentage of $^{125}$I-BSA$_{fd}$ that had been degraded at each time point was calculated as follows:-

$$\% \ ^{125}\text{I-BSA}_{fd} \text{ degraded} = \frac{\text{TCA-soluble radioactivity (c.p.m.)}}{\text{Total radioactivity (c.p.m.)}} \times 100$$

Both TCA-soluble and total counts were corrected for background radioactivity. The time-course of percentage degradation was plotted for each experiment.

Samples of the suspension were also removed at t=0 and at t=150min, to assess the amount of free, non-sedimentable $^{125}$I-BSA$_{fd}$ present in the preparation before and after the 2.5h incubation.

This gives a measure of the fraction of broken lysosomes in the preparation initially, and those formed during incubation. The samples were centrifuged at about 22,500g for 1h in order to pellet the lysosomes (and any lysed membranes). The supernatants and pellets were separated, and the pellets resuspended in sucrose (1ml). Serum (0.1ml) was added to the supernatants as a carrier protein then 0.5ml of aqueous TCA (20%,w/v) added to both the supernatants and resuspended pellets, to precipitate intact $^{125}I\text{-}BSA_{fd}$. The TCA precipitates were separated by centrifugation (1000g, 10min), resuspended in water (1ml) and assayed for radioactivity. The percentage of TCA precipitable radioactivity was used as a measure of lysosomal integrity. (A finding of 100% sedimentable, TCA-precipitable radioactivity would indicate complete absence of membrane lysis, and no release of intact $^{125}I\text{-}BSA_{fd}$.) The percentage was calculated as follows:-

$$\% \text{ intact lysosomes} = \frac{L}{L + S} \times 100$$

Where: L = TCA-insoluble fraction of the pelleted, lysosomally-associated radioactivity (c.p.m., corrected for background)

S = TCA-insoluble radioactivity associated with the supernatant after sedimenting the lysosomal pellet (c.p.m., corrected for background).

The validity of this method is discussed in Section 7.4.1.

## 7.3. RESULTS

The control rate of degradation (ie that observed in the absence of effectors) and the percentage of TCA-soluble material initially present varied between different lysosomal preparations, therefore the results could not be combined to give a mean time course of $^{125}$I-BSA$_{fd}$ degradation for each type of experiment. Hence, the figures shown are time-courses that were representative for each particular type of experiment.

### 7.3.1. Effect of Composition of Suspension Medium on the Rate of Intralysosomal $^{125}$I-BSA$_{fd}$ Degradation

Optimum conditions for protein degradation within lysosomes include an acidic intralysosomal pH. In the past much debate has surrounded the mechanism of acidification of lysosomes *in vivo*. A review by Reijngoud & Tager (1977) favoured a Donnan-type equilibrium, with negative lipoproteins within lysosomes maintaining a gradient of protons across the lysosomal membranes. However, strong evidence now exists for the presence of an $Mg^{2+}$-ATP-dependent proton pump, which actively pumps protons into lysosomes to maintain the pH gradient (eg Poole & Ohkuma, 1981; Ohkuma *et al.*, 1982).

The lysosomal suspension medium used by Ohkuma *et al.* (1982) gave good *in vitro* acidification of the lysosomes, whereas incubation in sucrose buffer alone caused a gradual increase in the intralysosomal pH. It was therefore decided to examine two different resuspension media for their effects on intralysomomal $^{125}$I-BSA$_{fd}$ degradation. The ATP medium used was similar to that suggested by Ohkuma *et al.* (1982). Unbuffered sucrose (0.25M), which is commonly used to provide osmotic protection for intact lysosomes (DeDuve *et al.*, 1955) was also tested, since this simple medium gave good

results in the work of Mego & McQueen (1965 a,b).

The time-courses of $^{125}I\text{-}BSA_{fd}$ degradation in lysosomes suspended in the two different media are shown in Figs. 7.1 and 7.2. The plots for both sucrose and ATP medium were generally slightly curved; the rate of degradation slowed after about 90-120min. Either there was no difference in the time-course of degradation (Fig. 7.1) or the rate observed in the ATP-medium was slightly lower than for sucrose alone (Fig. 7.2). The percentage of intact lysosomes in matched experimental preparations was similar for both types of resuspension media, both before and after the 150min incubation period.

### 7.3.2. Effects of Triton X-100 on the Rate of Degradation of $^{125}I\text{-}BSA_{fd}$

In order to assess whether leupeptin crosses the lysosomal membrane to inhibit proteolysis, it is essential to verify that the observed proteolysis occurs only within the isolated lysosomes. (If degradation of $^{125}I\text{-}BSA_{fd}$ continued after release of the lysosomal contents, any inhibition observed with leupeptin could arise from inhibition of extra-lysosomal degradation. Such inhibition would not require permeation of leupeptin through the lysosomal membrane.) Mego *et al.* (1967) demonstrated that in the presence of Triton X-100 (0.2%), or absence of osmotic protectors, the degree of inhibition depended on the volume of medium in which lysosomes were resuspended; extralysosomal hydrolysis did not occur when lysosomes from 1g liver were resuspended in 20ml sucrose solution. This inhibition was apparently caused by dilution of the enzyme-substrate mixture on release. The results were reported at both pH 5 and pH 7.3. At the latter pH, inactivation of the lysosomal cathepsins would also be expected to contribute to the inhibition.

For the experiments reported in this chapter the lysosome-enriched pellet was resuspended in medium to give about a 15%w/v suspension. The osmotically protective medium was usually either unbuffered or buffered to pH 7.0. It was important to check that these conditions did not give rise to extra-lysosomal proteolysis if the lysosomes became ruptured. Triton X-100 (0.1%) was therefore included in the incubation medium. A typical time-course of $^{125}I\text{-}BSA_{fd}$ degradation in the presence of the detergent is shown in Fig 7.3. No increase in the amount of TCA-soluble material over the 150min incubation was observed.

The percentage of intact lysosomes present (as measured via non-sedimentable intact $^{125}I\text{-}BSA_{fd}$) was determined at the beginning and end of these experiments. At the start of the experiment, immediately after the addition of Triton X-100, about 10 ± 1% of the lysosomes remained intact. This decreased to 4 ± 1% by the end of the 150min incubation period, (see Section 7.4.1 for possible explanations).

### 7.3.3. Effects of Leupeptin (10µg/ml) and of Ammonium Chloride (20mM) on the Rates of Intralysosomal Degradation of $^{125}I\text{-}BSA_{fd}$

The effects of these inhibitors were examined using a lysosome-enriched fraction suspended in either sucrose or ATP medium. (Results obtained using buffered sucrose will be described in Section 7.3.5.)

Ammonium chloride is frequently used to inhibit lysosomal protein degradation in cells and tissues (eg reviews by Dean et al., 1984; Seglen, 1983). It is thought to enter lysosomes uncharged then become protonated and trapped, causing vacuolization, an increase in lysosomal pH, and inhibition of proteolysis (Ohkuma & Poole, 1981;

Poole & Ohkuma, 1981). It has also been shown to inhibit degradation of $^{125}$I-BSA$_{fd}$ in isolated lysosomes (Reijngoud *et al.*, 1976). It was hoped to use this molecule as a 'positive control' to indicate that inhibition of intralysosomal proteolysis in this *in vitro* system was possible.

It was necessary to check whether the added inhibitors affected the integrity of the lysosomal membrane. In matched experiments, the difference between the initial and final percentage of re-sedimentable TCA-precipitable radioactivity (taken to indicate the fraction of lysosomes ruptured), after incubating lysosomes with leupeptin (10µg/ml) or ammonium chloride (20mM) was often less than or equal to that of the control (incubated without inhibitor). [The differences between control and inhibited lysosomes were similar for both sucrose and ATP-containing media.] This suggests that the inhibitors did not disrupt the lysosomal membrane.

Fig. 7.4 shows a typical time course of $^{125}$I-BSA$_{fd}$ degradation for the lysosomal preparation suspended in sucrose (0.25M) in the presence of (a), leupeptin, (10µg/ml); and (b), ammonium chloride (20mM). Leupeptin produced a marked inhibition of degradation but, contrary to expectation, the ammonium chloride did not appear to strongly inhibit intralysosomal $^{125}$I-BSA$_{fd}$ degradation.

The results obtained with ammonium chloride for these experiments were rather variable. The rate of degradation in the presence of ammonium chloride was generally similar to that in the matched control. However, occasionally it was observed to be slightly inhibited or even stimulated, compared with the control (see Figs. 7.5 a, b). (Leupeptin always gave rise to a marked inhibition, although the degree of inhibition, compared with the control, differed slightly with different preparations of lysosomes.) One possible explanation for this variability could be differences in

the pH of the unbuffered medium, or lack of important cofactors necessary for optimal degradative conditions in the various preparations. The experiments were therefore repeated using lysosomes suspended in the ATP medium, which standardizes such conditions. Typical results are shown in Fig. 7.6a (leupeptin 10µg/ml) and 7.6b (ammonium chloride, 20mM). Again the effect of ammonium chloride was variable. Often the rate of degradation of $^{125}$I-BSA$_{fd}$ was greater than that for the control over the first 60-90min, then decreased to a rate less than the control for the remainder of the incubation. Leupeptin consistently inhibited the rate of degradation fairly strongly, as in equivalent experiments in sucrose medium.

Curvature of the time-course was a feature of experiments that included the inhibitors, whether the lysosomes were suspended in sucrose or ATP medium. (This was unlikely to be due to increased rupture of the lysosomal membrane, which would itself inhibit degradation; see above). With leupeptin, the plot was generally strongly curved and tended towards a plateau with no further release of TCA-solubles after 90-120min. This may be partially attributable to a decreased amount of $^{125}$I-BSA$_{fd}$ degradation after this period, noted in Section 7.3.1. However, the marked differences between test and control time-courses suggest that the concentration of leupeptin within the lysosomes builds up slowly over the incubation period, to produce a gradual increase in the degree of inhibition.

### 7.3.4. Effect of Leupeptin Concentration on Intralysosomal Degradation of $^{125}$I-BSA$_{fd}$

The rate of $^{125}$I-BSA$_{fd}$ degradation was monitored in the presence of leupeptin at concentrations of 10, 25, and 100µg/ml. Since a similar inhibition was observed using either sucrose or ATP medium

with leupeptin at 10µg/ml (see section 7.3.3.), the higher concentrations of leupeptin were tested using lysosomes suspended only in sucrose solution (0.25M). Results are shown in Fig. 7.7.

The time-course of degradation could be considered to show two phases. During the first phase the rate of production of TCA-solubles depended on the amount of leupeptin present. During the second phase only a small rise in the percentage of TCA-solubles occurred, and this was similar for all leupeptin concentrations. The first phase (Fig. 7.7) lasted at least 60min and the duration appeared to increase as the concentration of leupeptin was decreased.

The pattern of results could be taken to suggest that a maximum degree of inhibition was reached at all concentrations of leupeptin, when the rate of increase in percentage TCA-solubles fell to approximately 1.5 ± 0.5% per hour.

### 7.3.5. Effect of pH on the Rate of Degradation of $^{125}$I-BSA$_{fd}$ and on the Effectiveness of the Inhibitors Leupeptin and Ammonium Chloride

In the previous section, it was noted that ammonium chloride did not strongly inhibit $^{125}$I-BSA$_{fd}$ degradation in isolated lysosomes. However, ammonium chloride is known to inhibit intralysosomal degradation of exogenous proteins in cells and tissues. This suggests that the ammonium chloride was not able to penetrate rapidly the lysosomal membrane under the conditions described in Section 7.3.4.

Uptake of weak bases into cells has been investigated by Ohkuma & Poole (1978), Poole & Ohkuma (1981), and Solheim & Seglen (1983). Ohkuma and Poole demonstrated that uptake of a base depended on the concentration of free, unprotonated base in the medium, which in turn was dependent on the pH of the medium. It was therefore decided to

study the effects of ammonium chloride and of leupeptin on the rate of $^{125}I\text{-}BSA_{fd}$ degradation in isolated lysosomes over the pH range 6.0-8.0.

The $pK_a$ of the ammonium ion is 9.245 (Atkins, 1978). The percentage of unprotonated $NH_3$ present at pH 6, 7, and 8 was calculated, using the Henderson-Hasselbalch equation, to be 0.06, 0.6, and 5.7% respectively. The $pK_a$ of leupeptin was not determined. The only ionizable group within the molecule is the guanidinium group of the arginal residue. In arginine, the pK of this group is 12.48, which means only a minute percentage would be unionized at pH 6-8 ($3x10^{-5}$ - $3x10^{-3}$%). However, the pK may not necessarily be the same for the guanidinium moiety in the leupeptin molecule because of the aldehyde rather than acid group adjacent, and because of the different hydration structures of leupeptin (Maeda et al., 1971, see Fig. 1.1).

Changes in the pH of the lysosomal suspension medium are reported to affect both the stability of the lysosomal membrane and the rate of intralysosomal protein degradation. These effects are discussed more fully in Section 7.4. The rate of $^{125}I\text{-}BSA_{fd}$ degradation was aways monitored both in the presence and absence of inhibitor to indicate whether any change in the rate of degradation was caused by the inhibitor itself or by the altered medium. The percentage of ruptured lysosomes was determined as described previously.

Fig. 7.8 shows the time-course of $^{125}I\text{-}BSA_{fd}$ degradation at pH 6.0 in the presence and absence of leupeptin (10µg/ml) or ammonium chloride (20mM). Neither inhibitor decreased the rate of degradation, suggesting that neither leupeptin nor ammonium chloride entered the lysosomes at a detectable rate at this pH.

At pH 7.0, the time courses of degradation were similar to those

observed previously (Figs 7.4, 7.5, 7.6). Ammonium chloride had little effect on the rate of degradation, whereas in the presence of leupeptin a marked inhibition occurred, as described in Section 7.3.4.

At pH 8.0, both ammonium chloride (20mM) and leupeptin (10µg/ml) inhibited intralysosomal $^{125}$I-BSA$_{fd}$ degradation (Fig. 7.10). Both inhibitors gave rise to approximately the same degree of inhibition. Inhibition was virtually complete after about 60-90min, with little further increase in the amount of TCA-solubles produced.

Fig. 7.11 shows the time-course of $^{125}$I-BSA$_{fd}$ degradation at each pH in the absence of inhibitors, determined using the same lysosomal preparation. During the first hour of incubation, the rates of degradation were very similar at each pH. (The duration of this close similarity between rates varied slightly for different lysosomal preparations, from between 1-2 hours). After this period, the rate of hydrolysis decreased at all pH's. The decrease at pH 8 was most marked, whereas at pH 6 only a slight decrease was observed.

This observation could possibly be explained by an increase in the amount of lysosomal lysis at the higher pH's. The percentage of resedimentable, TCA-precipitatable radioactivity (ie intact $^{125}$I-BSA$_{fd}$ within lysosomes), was therefore measured at the beginning and end of each incubation, and the difference between them (ie amount of lysis that occurred during incubation) calculated. For tissue incubated in the absence of any inhibitor, the decrease in the percentages of resedimentable intact $^{125}$I-BSA$_{fd}$ was 16 ± 4, 15 ± 4, and 18 ± 2, for pH 6, 7 and 8, respectively. Thus there was no marked difference in the degree of lysis at each pH. [This method of determining lysis could, however, have been affected by differences in affinity of $^{125}$I-BSA$_{fd}$ for the ruptured membranes at different pH's. Mego *et al.* (1967) reports that $^{125}$I-BSA$_{fd}$ binds to membranes

at acid pH, thus the percentage resedimentable $^{125}$I-BSA$_{fd}$ at pH 6 may be an overestimate of the amount of intact lysosomes.] The decreased rate of degradation observed at the higher pH's after 60min could not, therefore, be attributed to a greater amount of lysis.

## 7.4. DISCUSSION

### 7.4.1. General Features of the Mego & McQueen Method

This method of measuring the permeability of the lysosomal membrane towards certain lysosomal effectors offers a number of advantages. The method of detecting entry of the effector is comparatively direct (c.f. osmotic protection and vacuolization methods, which depend on secondary events such as osmotic swelling). The method is also convenient in the ease with which permeation is detected, and the experimental procedure is relatively simple. Another advantage is that the concentration of effector can be kept low, which is both less expensive and less likely to cause saturation of any transport systems. Finally, the concentration of the effector can be varied without affecting the osmotic pressue of the medium.

The method does, however, have certain limitations. Since $^{125}I\text{-}BSA_{fd}$ is the substrate most commonly used for loading the lysosomes (due to its exceptionally rapid clearance from the blood and comparatively slow degradation by lysosomes) the method is confined to studies of inhibitors or stimulators of proteases active against $^{125}I\text{-}BSA_{fd}$. These proteases have been characterised by Kooistra *et al.* (1982) and Mego (1984). Mego found that formaldehyde-denatured albumin, unlike albumin denatured by other methods or native albumin, was primarily degraded by leupeptin-sensitive thiol proteinases. Thus, $^{125}I\text{-}BSA_{fd}$ was suitable to use as a substrate to monitor the possible inhibition of intra-lysosomal proteolysis by extra-lysosomal leupeptin.

A disadvantage of this method of determining lysosomal permeability is that movement of the solute is only monitored in one direction, from outside to inside the lysosome. Since permeation mechanisms may be partially or totally uni-directional this gives

only a limited indication of the accessibility of lysosomal and non-lysosomal proteases to leupeptin. (If permeation occurred only from outside to inside the lysosome, leupeptin taken into the cell by pinocytosis may not have access to the cytosol. Conversely, if permeation occurred only from inside to outside the lysosome, uptake of leupeptin by permeation across the plasma membrane will not lead to entry into lysosomes.)

Another possible disadvantage is that many factors other than specific inhibitors or stimulators are known to affect the rate of protein degradation by isolated lysosomes. For example, prolonged incubation or incubation in buffers of certain pH may affect both the integrity and internal pH of the lysosomes. These factors must be taken into consideration when monitoring the effect of inhibitors or stimulators of proteolysis using isolated lysosomes, otherwise such effects may be mistakenly assumed to be caused by penetration of the inhibitor/stimulator into the lysosomes.

It is therefore important to measure lysosomal integrity, since rupture of the lysosomes affects the observed rate of $^{125}I\text{-}BSA_{fd}$ degradation. For the work reported in this chapter, the percentage of resedimentable, TCA-precipitable radioactivity was taken to indicate the percentage of intact lysosomes. This determination was quick and convenient, though some factors must be considered when interpreting the data. One problem is the possibility of any released $^{125}I\text{-}BSA_{fd}$ binding to lysed membranes, so becoming sedimented and counted as being within intact lysosomes. Mego *et al.* (1967) suggested that such binding only occurs at acid pH. Thus binding would not be expected to occur for most of the experiments reported in this chapter (although the presence of leupeptin or ammonium chloride might affect the degree of binding).

For experiments in which Triton X-100 was added to the

suspension medium, the percentage of resedimentable $^{125}I\text{-}BSA_{fd}$ decreased during the course of the incubation. This could suggest that lysis was not complete immediately after addition of the detergent, but progressed during incubation. Alternatively, intact $^{125}I\text{-}BSA_{fd}$ may have been adsorbed onto the lysed lysosomal membranes at the beginning of the experiment, but gradually became desorbed or the membrane became more fragmeted and thus non-pelletable, during incubation. (This possibility was suggested by Mego et al., 1967.)

The measurement of lysis may also be affected by the inhibition of proteolysis within the lysosomes. Disruption of lysosomes in which proteolysis is inhibited would result in the release of a greater amount of $^{125}I\text{-}BSA_{fd}$ than from non-inhibited lysosomes, giving rise to an overestimate of the degree of lysis (Mego, 1971).

In summary, the method developed by Mego & McQueen for isolating $^{125}I\text{-}BSA_{fd}$-loaded lysosomes allows the entry of protease effectors (inhibitors or stimulators) into lysosomes to be detected. It should give meaningful results provided other factors that could interfere with the rate of hydrolysis by the lysosomal preparation are monitored and taken into consideration when interpreting the data.

### 7.4.2. Results Obtained Using the Mego & McQueen Method

#### 7.4.2a. **Suspension Media**

Many different types of media have been used in the past to suspend lysosomes. Factors such as type and concentration of solute; type and concentration of ions; type, concentration and pH of buffers; as well as energy requirement and temperture of incubation, have all been varied to determine their effects on lysosomal integrity, intralysosomal pH, and proteolytic capacity.

The solute most often used for suspension media is sucrose, since this does not appear to cross the lysosomal membrane (Lloyd,

1969). It is usually used at a concentration of 0.25M, since this has been shown to give good osmotic protection (Lloyd, 1969). Concentrations of 0.2M (Mego & McQueen, 1965b) and 0.3M (Marzella et al., 1980) have also ben reported to give as good or better protection than 0.25M, and Sawant (1964) reported that 0.7M sucrose gave better osmotic protection than 0.3M. The total molarity of the 0.25M sucrose solution is often increased by the addition of other solutes such as ions, buffers and thiol agents.

The effects of ions on the stability of the lysosomal membrane has been investigated by several workers. Some reviews and reports on ion permeability and effects are given by Mego et al. (1967), Reijngoud & Taqer (1977), and Ohkuma et al. (1982). In the latter report a permeant anion such as $Cl^-$ and a divalent cation, $Mg^{2+}$ were found to be important for the activity of the lysosomal ATP-dependent proton pump. KCl and $MgCl_2$ are thus often included in resuspension media if ATP is to be added, and where maintenance of the intralysosomal pH is important.

Changes in the pH of an incubation medium, brought about by buffers, have several effects on lysosomes. Workers have measured the effect of pH on the stability of the lysosomal membrane; the data are somewhat conflicting, since different reports suggest isolated lysosomes are either stable over a range of pH, or are most stable at acid pH, or most stable at neutral pH. Thus, Lloyd (1971) noted that the permeability of lysosomes towards mannitol was unaffected by changes in pH over the range 5-7, and similarly Docherty & Hales (1979) found lysosomes to be stable for at least 30min over the pH range 5-8 (at 25°C). Sawant et al. (1964) found that lysosomal enzyme activity was most latent at neutral pH; the amount of free enzyme activity increased at acid pH. Lloyd (1969) also reported that a rise in free lysosomal enzyme activity occurred more rapidly

at pH 5.5, but only at 37°C, not at 25°C (at which temperature the lysosomes were stable). Ahlberg & Glaumann (1985) detected a decrease in lysosomal integrity after 30min incubation at 37°C at pH 5.5, but not at pH 7. Mego & McQueen (1965b), Mego et al. (1967) and Mego (1971) reported that lysosomal integrity decreased markedly on incubation at 37°C at pH 8 and 9, and was most stable at acid pH (pH 5). Likewise, Ahlberg et al. (1982) and Doherty & Hales (1979) also noted that the type of buffer used could affect the integrity of the membrane. The conflicting data most probably reflect the use of different incubation temperatures, types of buffers, and methods of assessing lysosomal integrity.

The pH of the incubation medium may also affect uptake of effectors into the lysosomes. This is mainly due to changes in the ionzation of the effector rather than changes in the property of the lysosomal membrane (provided that the integrity of the membrane is unaltered). Uptake of most amino acids and dipeptides was found to be more rapid at pH 7 than at pH 5 or 6 (Lloyd, 1971). It was suggested that this was probably due to pH-dependent changes in the ionic species of molecule present. Poole & Ohkuma (1981) presented evidence that the pH-dependence of uptake of weak bases into the lysosomes of macrophages (in situ) was due to changes in the amount of free unprotonated base present at different pH. [One report suggested that glucose uptake was affected by pH due to the ionization of a specific carrier for glucose facilitated-transport in the membrane (Docherty & Hales 1979).]

The presence of buffers in the suspension medium affects the rate of intralysosomal proteolysis in addition to affecting the lysosomal membrane and ionization of effectors. Mego & McQueen (1965a,b) found that maximal proteolysis occurred in buffer at pH 5. Mego (1971) further characterised the effect of buffers on

intralysosomal proteolysis. Buffers at pH 4, 7.3 and 8 produced a buffer-concentration dependent inhibition of proteolysis that was reversible, provided membrane rupture had not occurred, on transfer to pH 5. The rate of proteolysis in buffer at pH 5 was no different from that in unbuffered sucrose.

Several workers have added ATP (and necessary cofactors) to lysosomal suspension media in order to maintain a low intralysosomal pH and/or stimulate intralysosomal hydrolysis (eg Mego *et al.*, 1972; Okhuma *et al.*, 1982). Other examples of its use are given by Ahlberg & Glaumann (1985). This last paper also points out that some workers (eg Huisman *et al.*, 1974 a,b) have failed to demonstrate any effect of ATP. ATP was shown to enhance degradation of proteins captured by microautophagy (Ahlberg & Glaumann, 1985) but had no effect on the capture of Percoll by this mechanism.

In the work reported in this chapter, three different types of media were used to resuspend the lysosome-enriched pellet: sucrose (0.25M), buffered sucrose (0.25M sucrose containing 30mM phosphate buffer pH 6, 7 or 8) and 'ATP-medium' (containing ATP, $Mg^{2+}$, and KCl in sucrose solution, buffered to pH 7 with HEPES).

The integrity of the lysosomes was not greatly affected by the presence of buffer at pH 6, 7 or 8 on incubation at 25°C, which suports the reports of Lloyd (1969, 1971) and Docherty & Hales (1979). The rate of intralysosomal hydrolysis was only affected after about 60 minutes, at which time the rates of degradation decreased at all pH's, the largest decrease occurring at pH 8. Initially the rate of degradation was similar to that observed in unbuffered sucrose (although a direct comparison, using the same lysosomal preparation was not made). This is in marked contrast to results reported by Mego (1971) in which intralysosomal degradation was inhibited immediately on exposure to buffer. The origin of the

discrepancy in these observations is not known.

The ATP medium did not enhance the rate of $^{125}I\text{-}BSA_{fd}$ degradation. One possible explanation for this lack of effect was suggested by Ahlberg & Glaumann (1985). The procedure as used in this chapter for preparation of the lysosomal-enriched fraction would result in a fraction highly contaminated with mitochondria. These organelles have a large amount of ATP-ase activity associated with them, and hence ATP added to a suspension containing such impurities could be consumed by non-lysosomal particles.

### 7.4.2b. Effect of Ammonium Chloride.

Weak bases are frequently used to inhibit lysosomal protein degradation. Their mode of action was suggested by de Duve *et al.* (1974), who proposed that they entered lysosomes in the uncharged form then became protonated and trapped, leading to an increase in intralysosomal pH and osmotic swelling. More recently, Okhuma & Poole (1981) and Poole & Okhuma (1981), using macrophages, have been able to prove that entry of weak bases into lysosomes does indeed cause an increase in pH, and was dependent on the concentration of unprotonated base. The increase in intralysosomal pH occurred immediately on exposure of the cells to the base, and remained stable throughout prolonged incubation, even though the amount of base within the cells increased. Uptake of methylamine by hepatocytes has been characterised by Solheim & Seglen (1983) who found that two mechanisms were in operation, one energy-independent and one energy dependent; the latter was probably involved in lysosomal accumulation.

The work described above was carried out using intact cells. Few reports have been made of the effect of bases on isolated lysosomes. One such report, by Reijngoud *et al.* (1976), investigated

the effect of ammonium chloride and methylamine on intralysosomal $^{125}I\text{-}BSA_{fd}$ degradation using a method similar to that described in this chapter. A complex buffer at pH 6.2 and 7.5 was included in the 0.25M sucrose suspension medium, and a range of ammonium chloride concentrations from 0-100mM added. At either pH, inhibition of proteolysis was observed, the inhibition being greater at pH 7.5 than at 6.2. Only the initial 5-10 minutes of the time-course of degradation was used to measure the rate (since this declined after 10-15 minutes), and no measurement of lysosomal integrity was reported.

Results reported in the experimental section of this chapter were strikingly different. At pH 6 or 7 during the initial 60min of incubation with 20mM ammonium chloride (ie over the period monitored by Reijngoud et al., 1976), little difference in the rate of proteolysis relative to that of the matched control was noted. In some experiments there was even a suggestion of slight stimulation rather than inhibition. The percentage of free base ($NH_3$) in the medium at pH 6 and 7 was about 0.06 and 0.6% respectively, therefore the concentration of this permeant species was about 0.01 and 0.1mM, respectively. It is possible that these concentrations were too low to greatly affect the lysosomes. However, 20mM ammonium chloride produces a marked inhibition of intralysosomal proteolysis within intact cells when added to the incubation medium at pH 7.3 (eg Grinde & Seglen; 1980, Livesey et al., 1980), and an increase in lysosomal pH of about 2 units was detected by Poole & Ohkuma (1981) for macrophages incubated at pH 7.3. These inconsistencies are difficult to explain.

At pH 8, a marked inhibition of proteolysis occurred in the presence of ammonium chloride. Hence at this pH, it seems probable that sufficient inhibitor was able to pass across the lysosomal

membrane to increase the intralysosomal pH, thus inhibiting proteolysis. The concentration of free base in the medium at this pH was about 5.7% (1.2mM). Onset of inhibition was immediate, and the degree of inhibition appeared to be constant during the first 90min of incubation, when the control rate was linear. Thus, if any increase in the intralysosomal ammonium-ion concentration occurred over this period, it did not cause an increase in the degree of inhibition. This result is compatible with the results of Poole & Ohkuma (1981).

Ammonium chloride did not give rise to any increase in the degree of lysosomal rupture at any pH. Overall, it appeared to be slightly protective to lysosomal integrity (mean percentage lysis during incubation for all experimental conditions in the presence of ammonium chloride was 13%, compared with 16% for control incubations).

### 7.4.2c. Effects of Leupeptin.

Uptake of leupeptin into lysosomes has been studied, indirectly, by Neff *et al.* (1979) and by Ahlberg & Glaumann (1985). Neff *et al.* (1979) reported that leupeptin was unable to pass through the lysosomal membrane. This was based on the observation that no inhibition of degradation of endogenous proteins within isolated lysosomes was detected on incubation with leupeptin (10µg/ml) at pH 5 or 8. Conversely, Ahlberg & Glaumann (1985) found that degradation of proteins taken into lysosomes by microautophagy was inhibited by addition of leupeptin to the suspension medium. They reported that membranes were freely permeable to leupeptin, though this statement was based on an observation by Seglen *et al.* (1979) that no delay in the leupeptin-induced inhibition of proteolysis was apparent.

It should be noted that Neff *et al.* measured the effect of

extralysosomal leupeptin on the degradation of intralysosomal protein substrates, whereas in the work of Ahlberg & Glaumann, both inhibitor and substrate were extralysosomal. In view of these conflicting reports, a further study of the permeation of leupeptin into lysosomes was considered worthwhile.

The "Mego & McQueen method" was very suitable for the measurement of permeation of leupeptin (See Section 7.4.1.). Unlike the methods used by Neff et al., and Ahlberg & Glaumann, the protein substrate and degradation products were easily measured by gamma counting, and a large percentage of the protein present was degraded over the incubation period. Any changes in the amount of $^{125}I\text{-}BSA_{fd}$ degraded in the presence of leupeptin were therefore readily detectable.

Using this method, it was observed that leupeptin added exogenously gave rise to a marked inhibition of intralysosomal degradation of $^{125}I\text{-}BSA_{fd}$. Because the inhibitor did not cause lysosomal lysis (mean decrease in percentage of intact lysosomes during incubation with leupeptin, under all conditions tested, was 10 ± 4, whereas that for the controls was 16 ± 5), the inhibition must have been caused by leupeptin entering the lysosomes and inhibiting leupeptin-sensitive proteinases. The rate of proteolysis in the presence of leupeptin appeared to be dose-dependent during the initial period of incubation (about 60min). However, after this period the rate of proteolysis decreased to a common minimum rate at all leupeptin concentrations. This suggests that during the initial period the concentration of leupeptin in the lysosomes was not sufficient to induce maximal inhibition, but inhibition increased at a rate proportional to the leupeptin concentration in the medium. Once maximal inhibition was achieved (ie all leupeptin-sensitive enzymes were fully inhibited) the same low rate of degradation

(presumably mediated by non-leupeptin-sensitive enzymes) was achieved at all concentrations of leupeptin. Alternatively, leupeptin could reach a steady-state concentration within the lysosomes, either as a result of inactivation of the inhibitor or by permeation back out of lysosomes such that the rate of influx was equal to the rate of efflux. However this would not explain the similarity between the rates for diferent concentrations of leupeptin (Fig. 7.7) unless the steady-state concentration was greater than the amount required to fully inhibit the leupeptin-sensitive enzymes.

The pH of the medium affected the entry of leupeptin into the lysosomes. At pH 6, no leupeptin-induced inhibition occurred, whereas at pH 7 and 8 a strong inhibition was observed. This suggests that entry of leupeptin was dependent either on some pH-sensitive change in the leupeptin molecule or on a pH-induced change in a transport mechanism in the membrane.

A change in the ionization of leupeptin between pH 6 and 7 was not expected, because the guanidinium group of the arginyl residue remains charged at either pH (only a slightly greater percentage is charged at pH 6). Possibly the structure of leupeptin altered by cyclization involving the guanidinyl group (Maeda et al., 1971) or the pH dependence of uptake reflects a change in the ionization of a membrane transport component. Alternatively a pH-dependent change in the rate of uptake by microautophagy may be possible. Ahlberg & Glaumann (1985) reported that degradation of methaemoglobin by microautophagy was more rapid at pH 5.5 than at pH 7, but it was not made clear whether or not the rate of uptake, as well as of degradation, was affected by the lower pH. The rate of degradation depended on the type of protein used, which suggests that binding to the lysosomal membrane could be important. If microautophagy was the main route of entry of leupeptin in lysosomes, the effect of pH on

uptake of leupeptin could be explained by a difference in affinity of leupeptin for the membrane at pH 6 compared with pH 7 and 8 (and/or a difference in the rate of microautophagy).

The presence of ATP had no effect on the degree of inhibition observed with leupeptin. (Possible reasons for this lack of effect were discussed in Section 7.4.2a)

The method used in this chapter to study the permeation of a proteinase inhibitor has also been used to study the permeation of a stimulator of proteolytic enzymes, glutathione (Mego, 1984). Reduced glutathione (glutamyl-cyteinyl-glycine, GSH) acts both by reducing disulphide bonds in proteins during degradation (Mego, 1984; Kosistra et al., 1982) and as an essential cofactor for thiol proteinases, (eg Barrett 1977). Mego (1984) demonstrated that, although GSH was capable of stimulating proteolysis in a lysed tritosome extract (its effect being saturatable at 10-20mM), it had no effect on the degradation of $^{125}$I-BSA$_{fd}$ within intact liver lysosomes. Conversely, it did stimulate the same process within intact kidney lysosomes, its effect being saturable at 2mM. It was suggested that GSH could not permeate liver lysosomes, but a saturable system might exist in kidney lysosomes to facilitate entry of the tripeptide.

Permeation of another tripeptide, glycyl-glycyl-glycine, was investigated by Lloyd (1971), using the osmotic protection method. It was found to cause lysosomal rupture, which suggested that it was able to permeate the membrane. Rupture occurred slowly, the most rapid rate occurring at pH 7. It was suggested that the pH effect was due to the formation of greater amounts of unprotonated species at pH 7 than at the other pH's tested (ie 5 and 6). The possibility of intralysosomal digestion of the peptide was not discussed. Degradation of the tripetide could lead to an even greater osmotic

pressure inside the lysosomes, if the rate of permeation of digestion products out of the lysosome was slower than the rate of influx of the tripeptide. Slower digestion at pH 7 could thus explain the more rapid membrane rupture at this pH.

The mechanism of uptake and lysosomal membrane permeability towards peptides were investigated by Ehrenreich & Cohn (1969), using the vacuolization method. Only the D-isomers of certain peptides caused vacuolization of macrophage lysosomes. (The L-isomers of the same peptides did not induce vacuolation, presumably because of their rapid rate of degradation). The tripeptide $(D\text{-}Ala)_3$ appeared to be taken into the macrophage by pinocytosis, and to accumulate within lysosomes, indicating that it was not able to permeate either the plasma or lysosomal membranes.

The pentapeptide pepstatin has been investigated by Furuno *et al.* (1983) in an attempt to determine the effect of pepstatin on proteolysis. In order to load lysosomes with pepstatin it was necessary to couple the peptide to a degradable glycoprotein (asialofetuin) that was taken up by adsorptive pinocytosis into rat liver hepatocyte lysosomes following intravenous injection. The glycoprotein was then degraded by lysosomal enzymes, leaving lysosomes loaded with free pepstatin. When lysosomes were isolated 3h after injection of the complex (3mg) and incubated *in vitro* at 37°C in 0.25M sucrose, about 60% of the intralysosomal pepstatin was released during the 60min incubation period. The rate of release was virtually constant during this period, and was much greater than the rate of release of a marker enzyme, β-glucuronidase. It was suggested that, although pepstatin was thought to be incapable of crossing the plasma membrane, it was able to leak out of loaded lysosommes because of the non-ionic form in which it existed at the low intralysosomal pH.

In summary, the literature reports on the permeability of lysosomal membranes suggest that some, but not all, small peptides are able to cross the membrane. Some of the variation in published results for leupeptin may have been caused by differences in methodology. Results reported in this chapter from Mego & McQueen style experiments show that leupeptin was able to enter liver lysosomes and inhibit intralysosomal proteolysis. The magnitude of the induced effects was shown to be dependent on both the leupeptin concentration and the pH of the medium, but not on the presence of ATP.

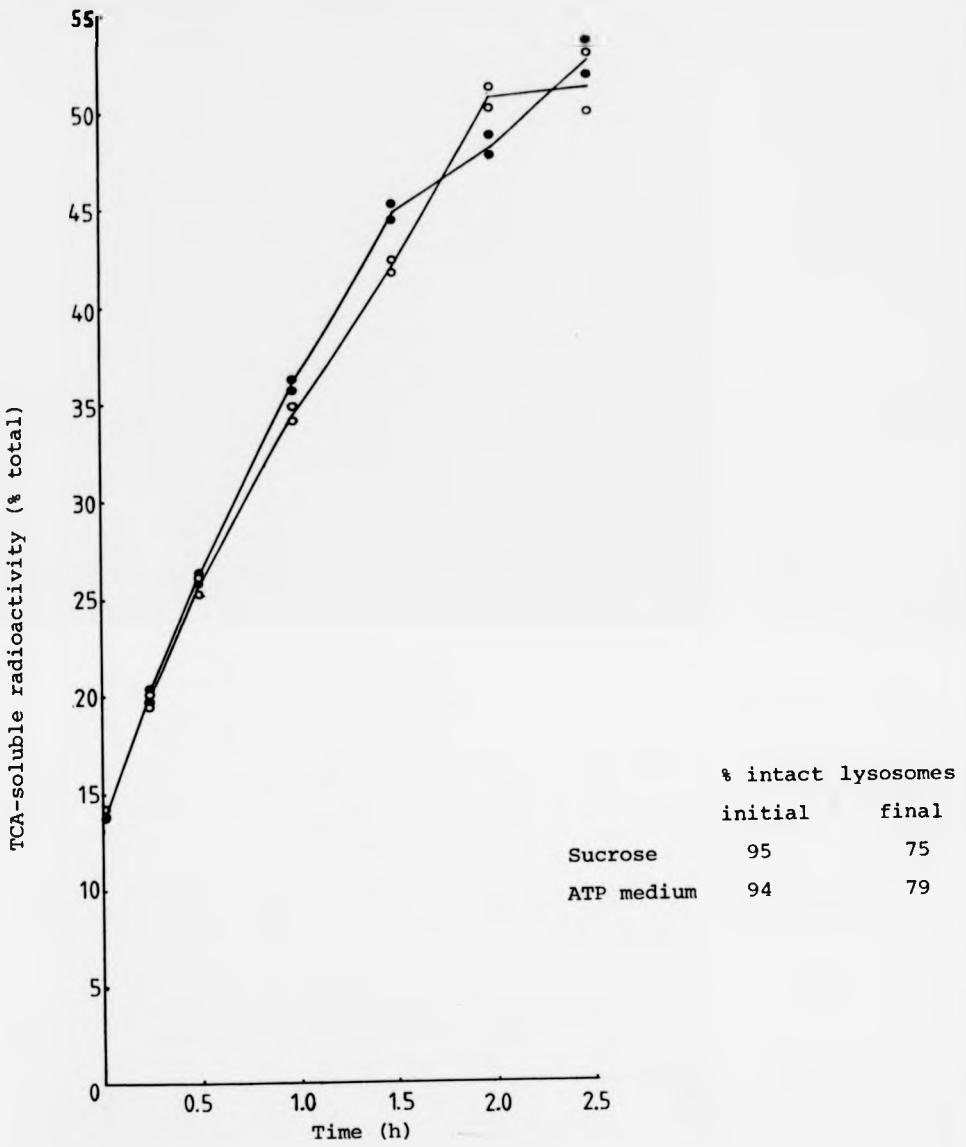
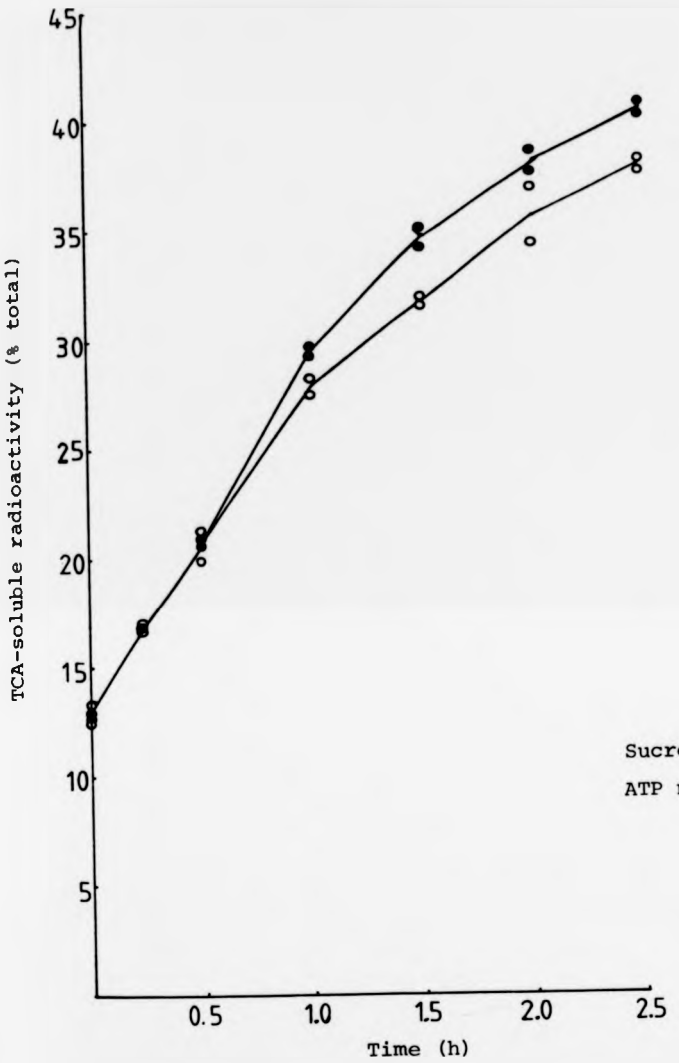



Fig. 7.1 Time-Course of $^{125}$I-BSA$_{fd}$ Degradation Within Isolated Lysosomes Suspended in Sucrose and ATP Media

$^{125}$I-BSA$_{fd}$-loaded lysosomes were prepared and incubated in sucrose (0.25M) or medium containing ATP as described in Section 7.2.1. Appearance of TCA-soluble $^{125}$I-BSA$_{fd}$ degradation products was monitored as described in Section 7.2.2. The graph shows typical results, in which no difference was observed between the rate of $^{125}$I-BSA$_{fd}$ degradation in the two suspension media.




| | % intact lysosomes | |
|---|---|---|
| | initial | final |
| Sucrose | 92 | 87 |
| ATP medium | 92 | 80 |

• Sucrose (0.25M)

∘ ATP medium

Fig. 7.2 Time-Course of $^{125}$I-BSA$_{fd}$ Degradation Within Isolated Lysosomes Suspended in Sucrose and ATP Media

The experimental details were identical to those described for Fig. 7. The graph shows a typical result in which ATP had a slight inhibiting effect on $^{125}$I-BSA$_{fd}$ degradation.

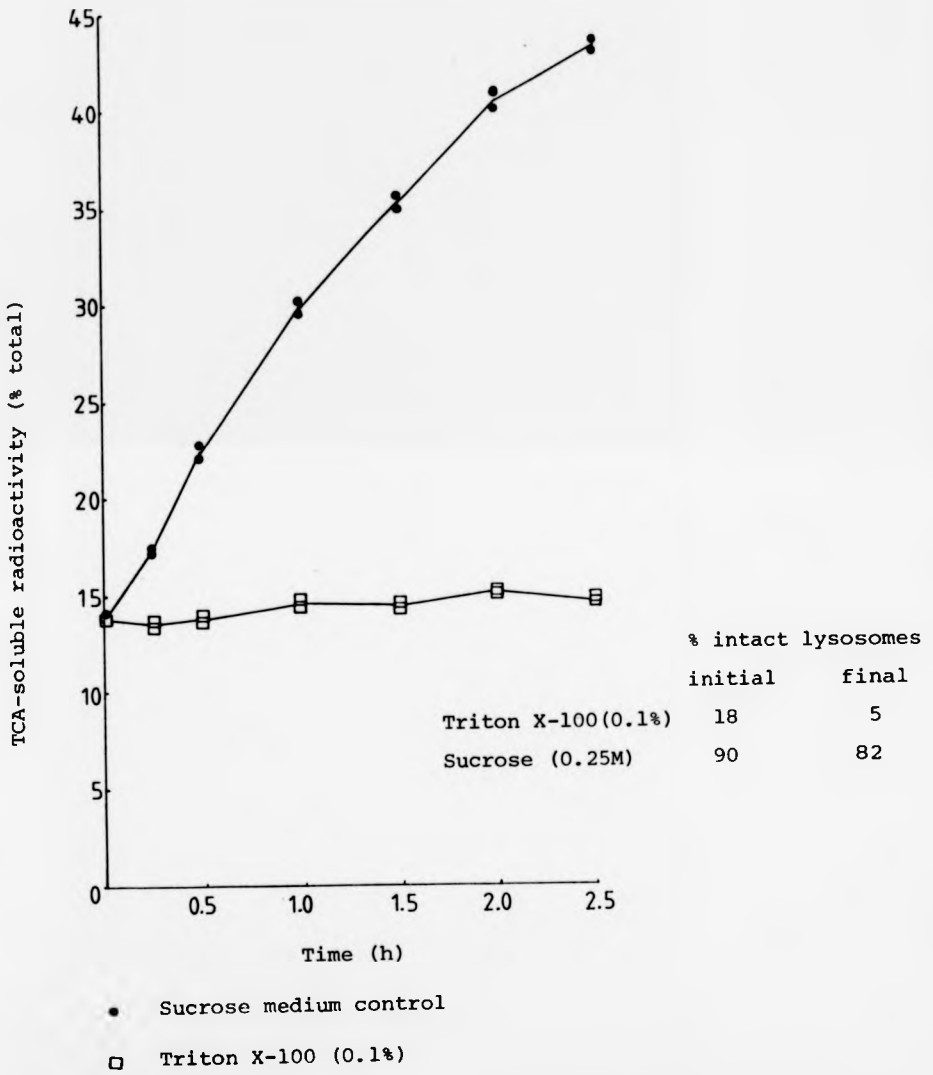



Fig. 7.3 Time-Course of $^{125}$I-BSA$_{fd}$ Degradation by Disrupted Lysosomes

$^{125}$I-BSA$_{fd}$-loaded lysosomes were prepared and suspended in sucrose (0.25M) in the presence and absence of Triton X-100 (0.1%) then the production of TCA-soluble material monitored, as described in Sections 7.2.1 and7.2.2. The graph shows a typical result.

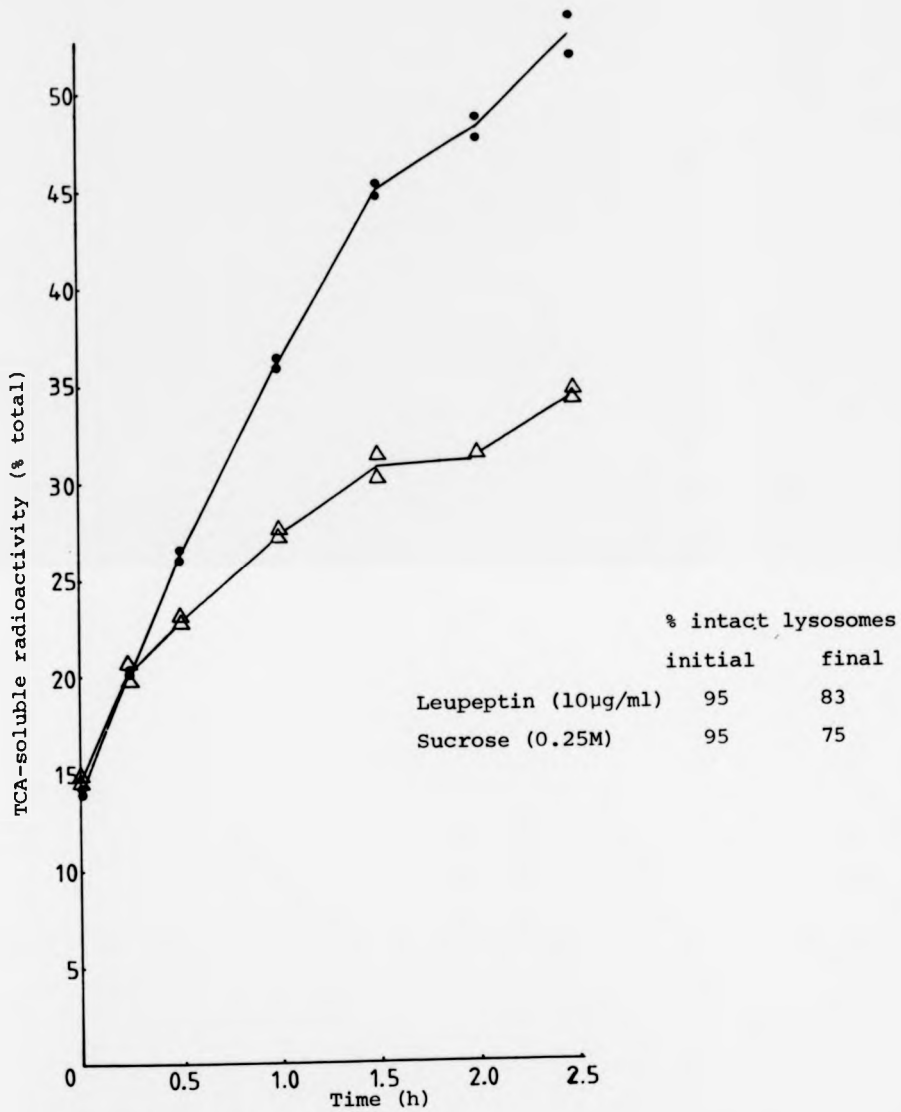



Fig. 7.4a Time-Course of $^{125}$I-BSA$_{fd}$ Degradation Within Isolated Lysosomes Incubated in Sucrose (0.25M) containing Leupeptin

Production of TCA-soluble radioactivity by $^{125}$I-BSA$_{fd}$ loaded lysosomes suspended in sucrose (0.25M) in the presence and absence of leupeptin (10 µg/ml) was monitored as described in Sections 7.2.1 and 7.2.2. The graph shows a typical result.

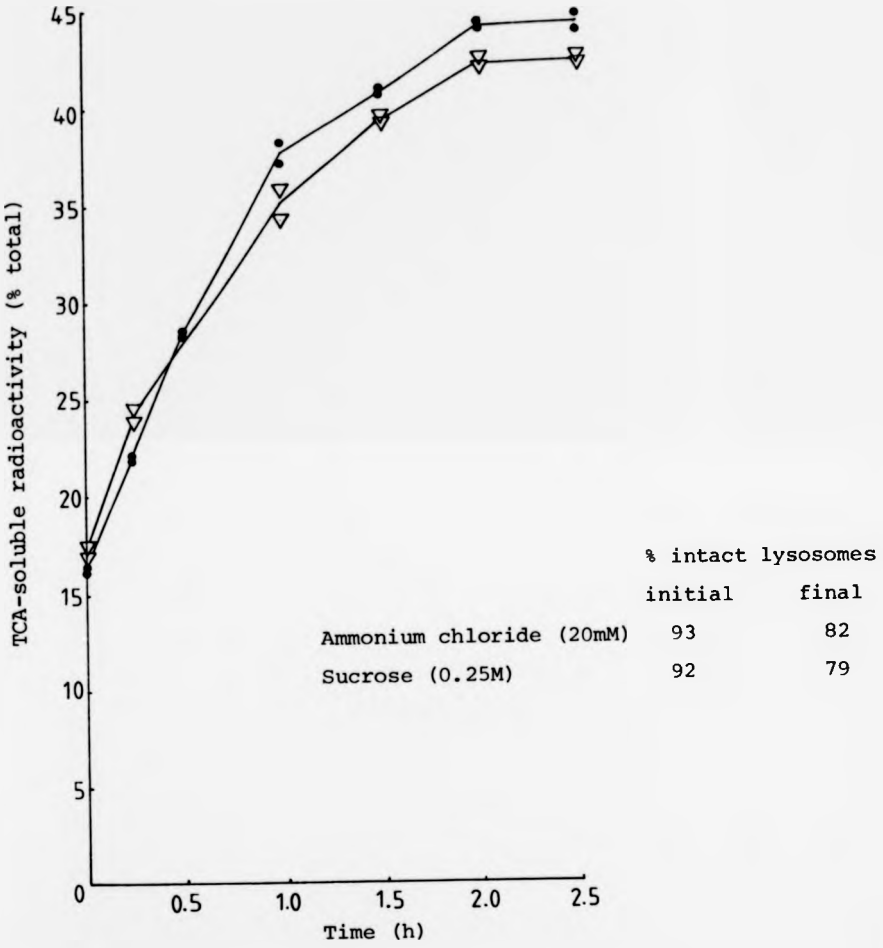



Fig. 7.4b Time-Course of $^{125}$I-BSA$_{fd}$ Degradation Within Isolated Lysosomes Incubated in Sucrose (0.25M) containing Ammonium Chloride

$^{125}$I-BSA$_{fd}$-loaded lysosomes were prepared and suspended in sucrose (0.25M) in the presence and absence of ammonium chloride (20mM), the the production of TCA-soluble radioactivity monitored. Experimental details are given in Sections 7.2.1 and 7.2.2. The graph shows a typical result in which little difference was observed in the rate of $^{125}$I-BSA$_{fd}$ degradation in the presence and absence of ammonium chloride.

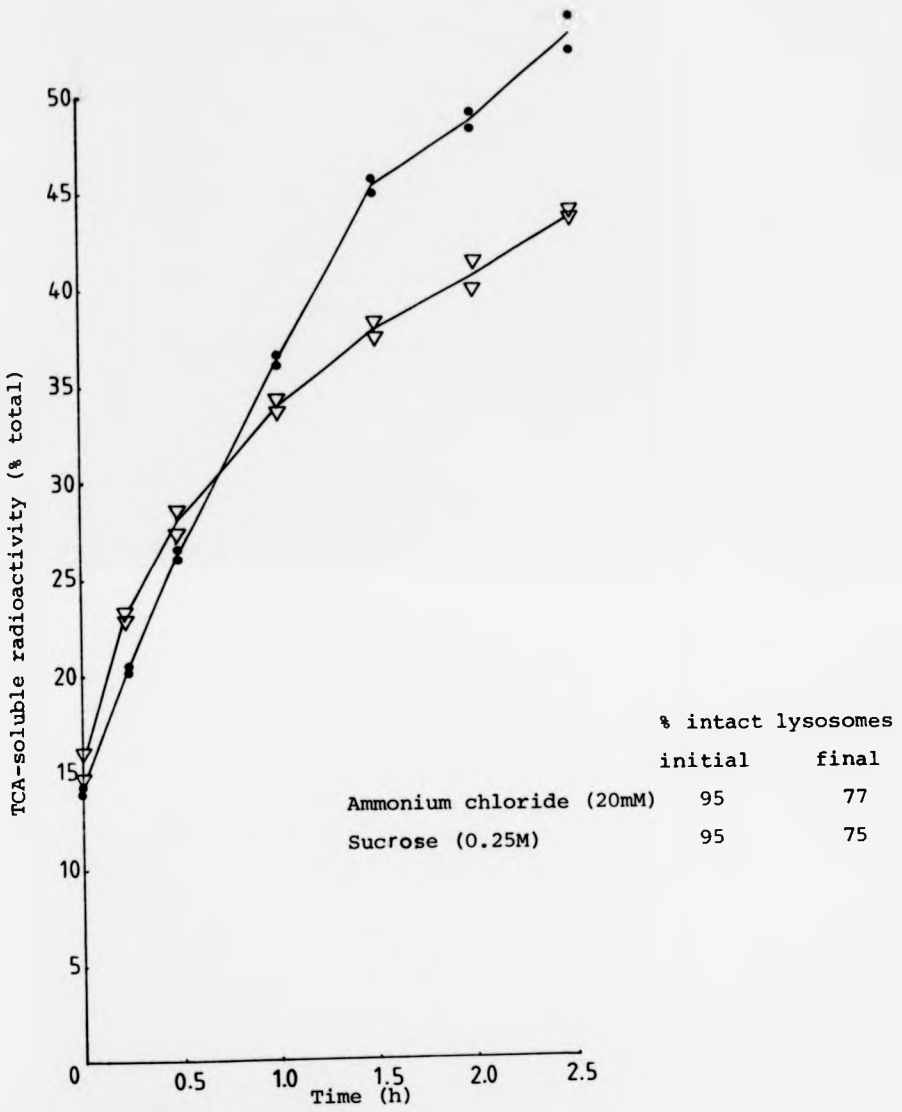



Fig. 7.5a Effect of Ammonium Chloride on the Time-Course of $^{125}$I-BSA$_{fd}$ Degradation

Experimental details were identical to those described for Fig. 6.4b. The graph shows a typical result in which $^{125}$I-BSA$_{fd}$ degradation was slightly inhibited by ammonium chloride.

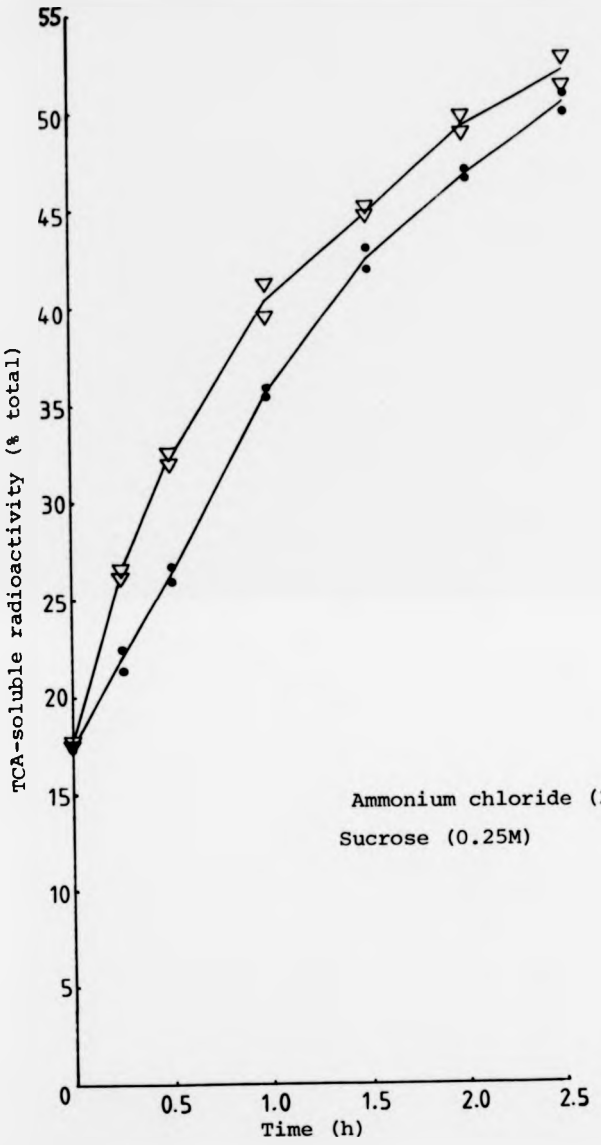



Fig. 7.5b Effect of Ammonium Chloride on the Time-Course of $^{125}I\text{-}BSA_{fd}$ Degradation

Experimental details were identical to those described for Fig. 7.4b. The graph shows a typical result in which $^{125}I\text{-}BSA_{fd}$ degradation was slightly stimulated by ammonium chloride.

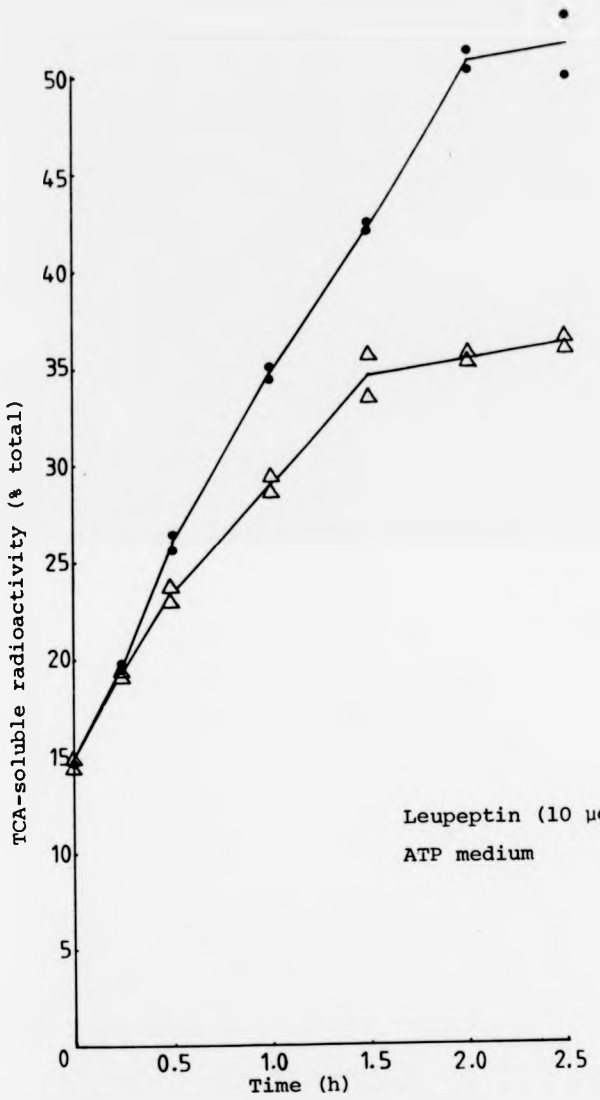
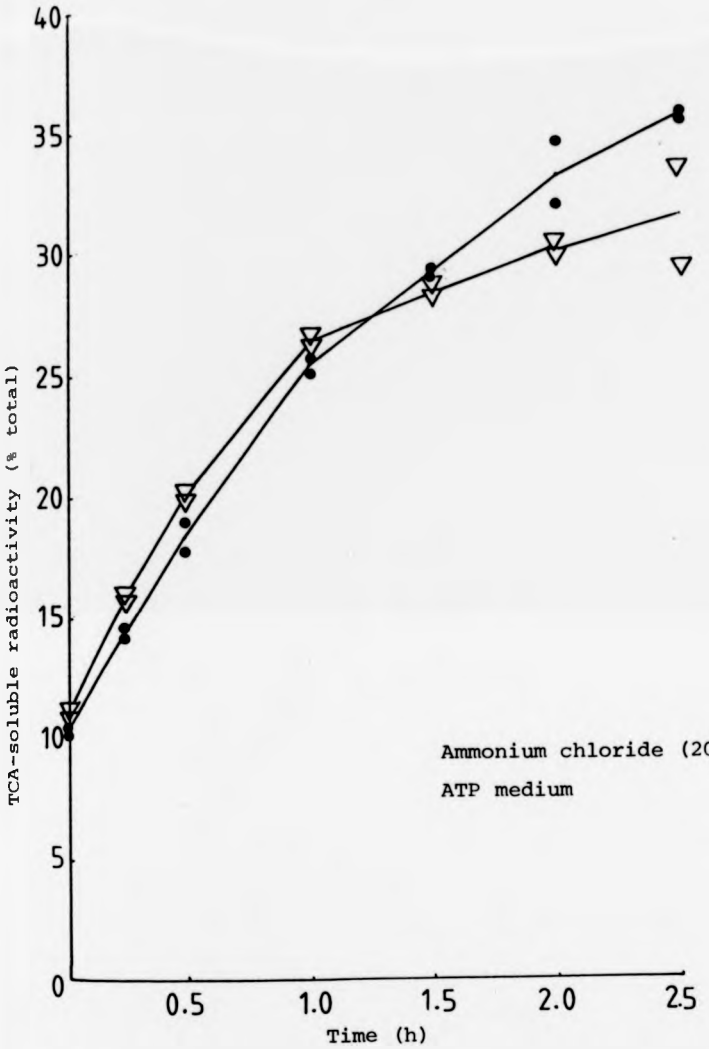



Fig. 7.6a Time-Course of $^{125}$I-BSA$_{fd}$ Degradation Within Isolated Lysosomes Incubated in ATP Medium containing Leupeptin

Production of TCA-soluble radioactivity by $^{125}$I-BSA$_{fd}$-loaded lysosomes suspended in ATP-medium in the presence and absence of leupeptin (10 µg/ml) was monitored as described in Sections 7.2.1 and 7.2.2. The graph shows a typical result.




| | % intact lysosomes | |
|---|---|---|
| | initial | final |
| Ammonium chloride (20mM) | 89 | 82 |
| ATP medium | 92 | 80 |

● ATP medium control

▽ Ammonium chloride (20mM)

Fig. 7.6b Time-Course of $^{125}$I-BSA$_{fd}$ Degradation Within Isolated Lysosomes Incubated in ATP Medium containing Ammonium Chloride

Production of TCA-soluble radioactivity by $^{125}$I-BSA$_{fd}$-loaded lysosomes suspended in ATP medium in the presence and absence of ammonium chloride (20mM) was monitored as described in Sections 7.2.1 and 7.2.2. The graph shows a typical result.

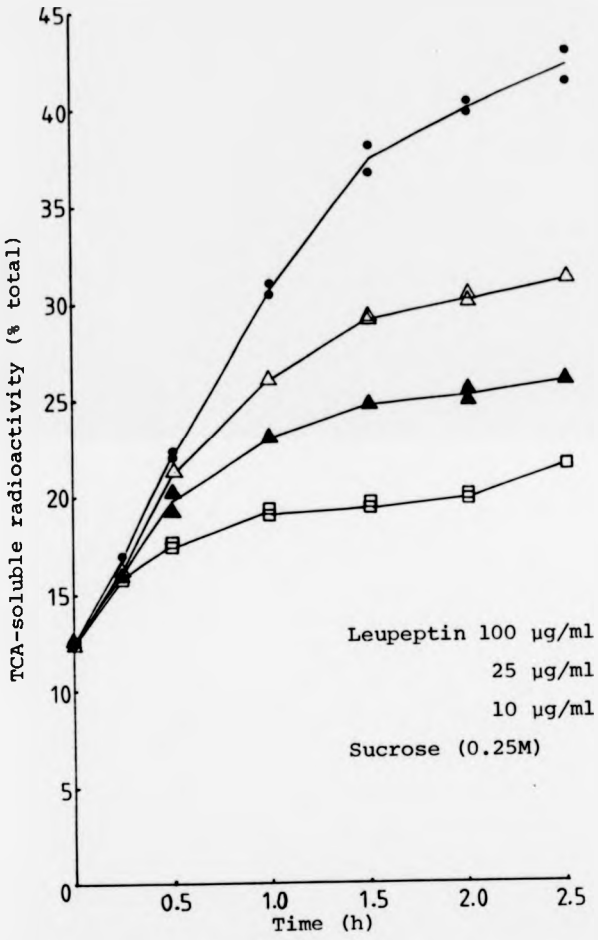



Fig. 7.7 Effect of Leupeptin Concentration on the Time Course of $^{125}$I-BSA$_{fd}$ Degradation Within Lysosomes

Production of TCA-soluble radioactivity by $^{125}$I-BSA$_{fd}$-loaded lysosomes suspended in sucrose (0.25M) in the presence of various concentrations of leupeptin was monitored as described in Section 7.2.1 and 7.2.2. The graph shows a typical result.

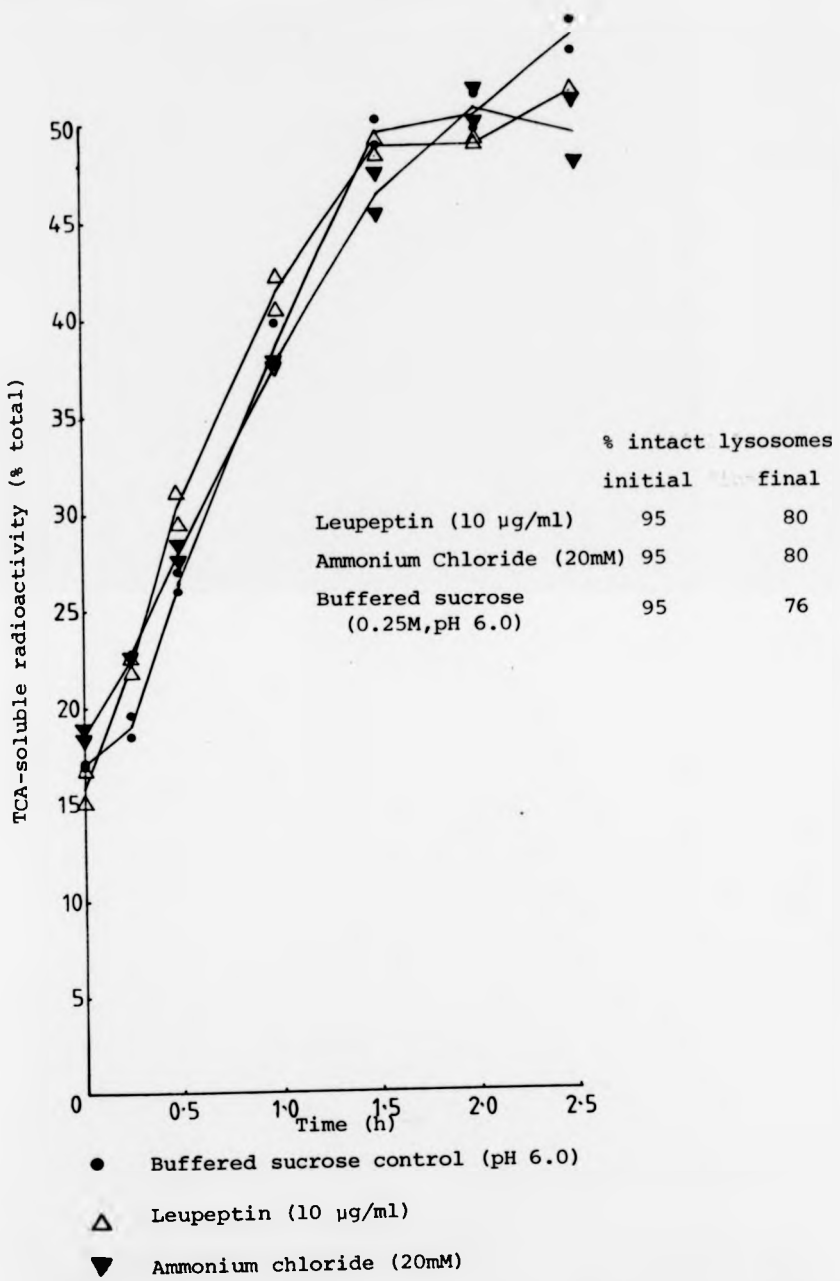



Fig. 7.8 Effect of Ammonium Chloride and Leupeptin on Degradation of $^{125}$I-BSA$_{fd}$ Within Lysosomes Incubated at pH 6.0

$^{125}$I-BSA$_{fd}$-loaded lysosomes were prepared and suspended in buffered sucrose (0.25M, pH 6.0) containing either leupeptin (10 µg/ml) or ammonium chloride (20mM). Production of TCA-soluble radioactivity was monitored, as described in Sections 7.2.1 and 7.2.2. The graph shows typical results.

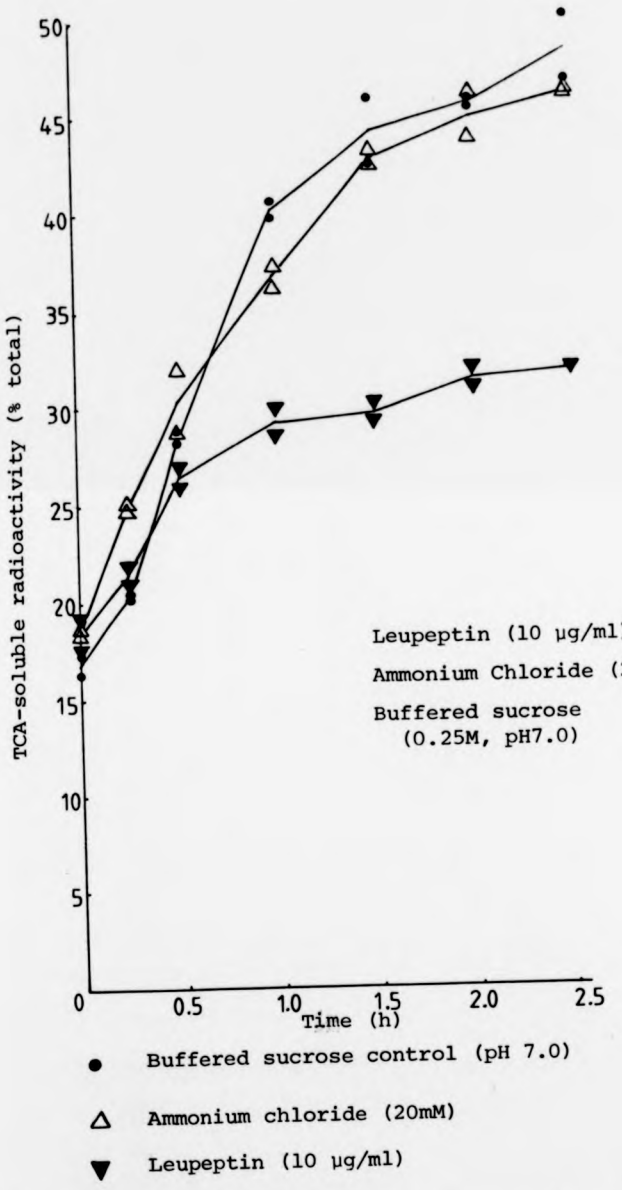



Fig. 7.9 Effect of Ammonium Chloride and Leupeptin on Degradation of $^{125}$I-BSA$_{fd}$ Within Lysosomes Incubated at pH 7.0

Experimental details were as described for Fig. 7.8, except that lysosomes were suspended in sucrose buffered to pH 7.0. The graph shows a typical result.

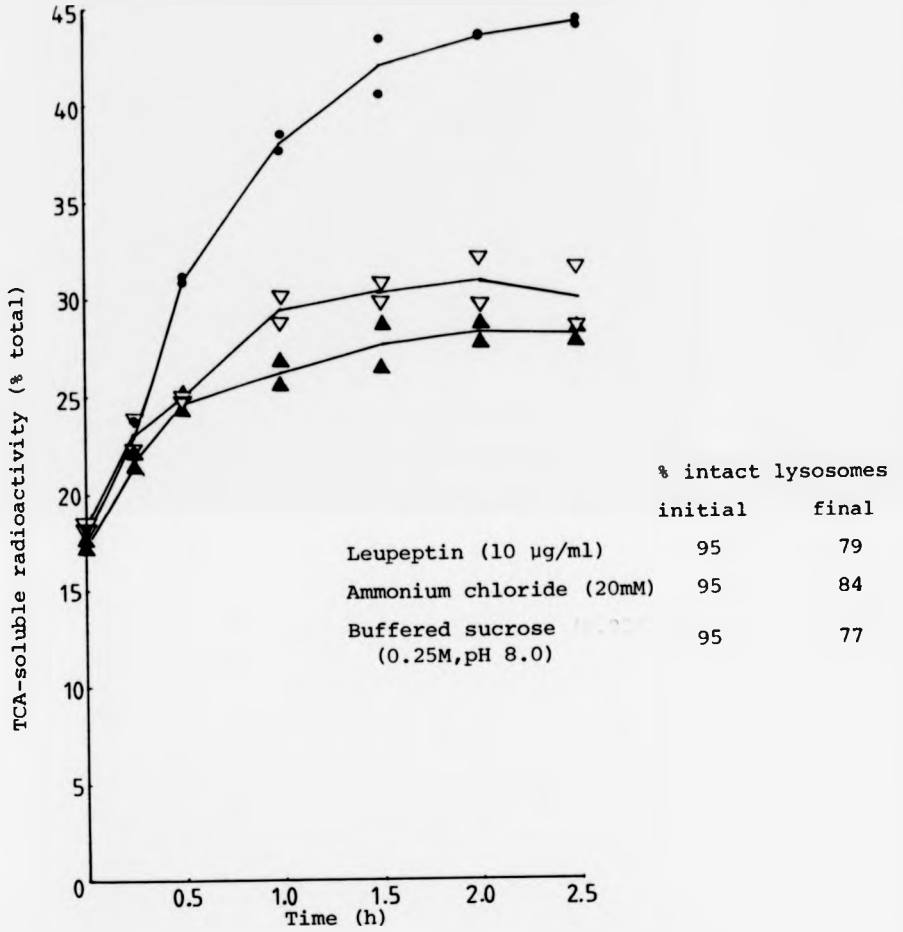



Fig. 7.10 Effect of Ammonium Chloride and Leupeptin on Degradation of $^{125}$I-BSA$_{fd}$ Within Lysosomes Incubated at pH 8.0

Experimental details were as described for Fig. 7.8 except that lysosomes were suspended in sucrose buffer to pH 8.0. The graph shows a typical result.

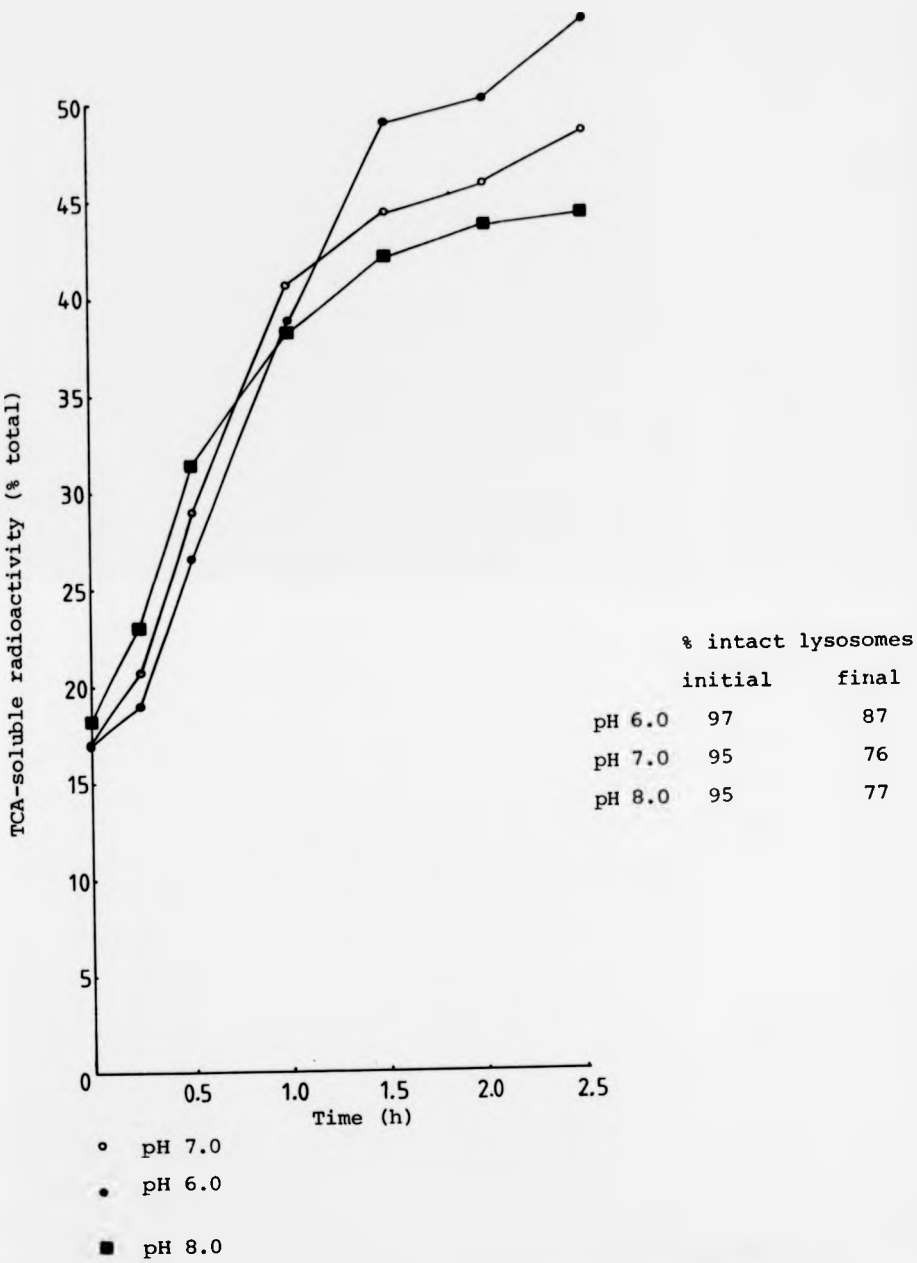



Fig. 7.11 Effect of Incubation pH on the Time-Course of $^{125}$I-BSA$_{fd}$ Degradation Within Isolated Lysosomes

$^{125}$I-BSA$_{fd}$-loaded lysosomes were incubated in sucrose buffered to pH 6.0, 7.0 and 8.0 in the absence of leupeptin and ammonium chloride, as controls for experiments described in Figs. 7.8, 7.9 and 7.10. The time course of $^{125}$I-BSA$_{fd}$ degradation at each pH is shown.

# CHAPTER 8

# CATHEPSIN B+L ASSAY

## 8.1. INTRODUCTION.

Although many studies have been made of the effects of leupeptin on endogenous and exogenous protein degradation (eg Knowles *et al.*, 1981; Wildenthal *et al.*, 1980; Dean, 1980) comparatively few investigators have attempted to correlate directly the observed degree of inhibititon of protein degradation with the degree of inhibition of intracellular proteinases by leupeptin.

Neff *et al.* (1979) measured the cathepsin B activity of hepatocytes after incubation with leupeptin (10µg/ml) for 4 hours, by using the specific substrate carbobenzoxy-alanyl-arginyl-arginyl-4-methoxy-B-napthlylamine. Cathepsin B was inhibited by 25-50%, whereas endogenous 'long-lived' protein degradation was inhibited by only 20%. No other leupeptin-sensitive cathepsins were assayed in this investigation.

Kominami *et al.* (1980) measured the inhibition of cathepsins B and L (although a non-specific substrate was used for the assay of cathepsin L) in a rat liver lysosome fraction after administration of leupeptin to the rat intraperitoneally. Cathepsin B was inhibited by 80% and cathepsin L by 40%, but no inhibition of degradation of total soluble proteins was detected.

Shaw & Dean (1980) investigated the effects of another inhibitor, benzyloxycarbonyl-phenylalanyl-alanyl-diazomethane (Z-Phe-Ala-$CHN_2$) on protein degradation and on cathepsin B activity in macrophages, using the substrate benzyloxycarbonyl-arginyl-arginyl-2-napthylamide. Cathepsin B was inhibited by 95% after 90min exposure to Z-Phe-Ala-$CHN_2$, compared with only about 30% inhibition of protein degradation after 24h exposure.

The principal aim of the work described in this chapter was to

measure the time-course of inhibition of leupeptin-sensitive enzymes in rat yolk sacs previously exposed to leupeptin, and to compare these results with those for the time-course of inhibition of degradation of an exogenous protein (monitored in Chapter 3).

The hydrolytic activity of yolk sacs that had been treated in different ways before homogenization was measured using carbobenzoxy-phenylalanyl-arginyl-aminomethylcoumarin (Z-Phe-Arg-AMC) as the substrate. The homogenates were derived from: unincubated tissue; unincubated tissue to which known amounts of leupeptin had been added; tissue that had been incubated in the absence of leupeptin; and tissue that had been incubated in the presence of leupeptin.

The substrate chosen for determining enzyme activity, Z-Phe-Arg-AMC, is reported to be an excellent substrate for assay of cathepsins L and B (Barrett & Kirschke, 1981), both of which are very sensitive to inhibition by leupeptin. A survey of the available literature on proteinases (eg Barrett & McDonald, 1980) suggested that no other intracellular proteinases were likely to hydrolyse this substrate. (Z-Phe-Arg-AMC is also a good substrate for trypsin and trypsin-like enzymes, and for tissue and plasma kallilreins. However, such enzymes are generally extracellular or confined to glandular tissue, hence were unlikely to be present within the yolk sac.) It is possible that some intracellular leupeptin-sensitive enzymes may not be capable of hydrolysing this substrate, in which case the total effect of leupeptin on proteinases would be underestimated.

Cathepsin B is a cysteine proteinase with a mol. mass of about 29,000. With synthetic substrates, it shows maximal activity at pH 6.0; activity decreases sharply at pH 7.0 due to irreversible inactivation. Many substrates (both synthetic peptides and natural

proteins) are known to be cleaved by cathepsin B, the main requirement for such substrates being an arginyl residue in the $P_1$ position and a phenylalanyl or basic residue at the $P_2$ site. The activity of cathepsin B in a cell extract is occasionally seen to increase during storage at -20°C (possibly due to inactivation of endogenous inhibitors). The kinetic constants for hydrolysis of Z-Phe-Arg-AMC by cathepsin B are: $K_m$ = 0.15mM; $k_{cat}/K_m$ = 9900 x $10^3$ $sec^{-1}M^{-1}$, specific activity = 238 units/μmol.

Cathepsin L is also a cysteine proteinase, with a pH optimum of 5.0-5.5 (though it is active over a fairly wide range of pH, including pH 7). It is a glycoprotein with a mol. mass in the range 21,000 -24,000. It is considered to be the most powerful of the lysosomal proteinases, with a higher specific activity against protein susbstrates than cathepsin B (Barrett & Kirshke, 1981). It is capable of degrading many proteins, but its activity against synthetic substrates is limited. Z-Phe-Arg-AMC is an exception, being a very good substrate. (With this substrate the enzyme shows a $K_m$ of 0.007mM; $K_{cat}/K_m$ = 3700 x $10^3$$sec^{-1}$ $M^{-1}$ and a specific activity 1200 units/μmol.)

The method of assay used was based on that described by Barrett (1980). It was hoped that the experiments described in this chapter would allow several factors to be investigated. In summary, these were:-

i) The cathepsin B+L activity of homogenates of unincubated 17.5 day rat yolk sacs.

ii) The effect of incubation of yolk sacs in medium 199 on the observed cathepsin B+L activity of the tissue.

iii) The amount of leupeptin required to induce a given degree of inhibition of the cathepsin B+L activity in an unincubated yolk-sac homogenate.

iv) The time-course of inactivation of cathepsin B+L on incubating yolk sacs with leupeptin, compared with the time course of inhibition of $^{125}I\text{-}BSA_{fd}$ degradation measured in Chapter 3. (In particular, it was desirable to try to assess whether the incomplete inhibition of $^{125}I\text{-}BSA_{fd}$ degradation observed in Chapter 3 was caused by an incomplete inhibition of cathepsins B and L or whether it was the result of degradation via leupeptin-insensitive proteinases such as cathepsin D.)

v) An attempt was made to quantify the amount of leupeptin entering the tissue during incubation with leupeptin by comparing the degree of inhibition observed with that obtained on adding leupeptin to unincubated yolk-sac homogenates. Results could then be compared with those obtained in Chapter 5, in which the amount of leupeptin extracted from tissue was quantified using a trypsin-based assay method.

## 8.2 MATERIALS AND METHODS

### I. MATERIALS

**Reagents**

Reaction buffer — A phosphate buffer, pH 6.0, containing EDTA and cysteine was used. $KH_2PO_4$ (23.93g) $Na_2HPO_4$ (3.41g) and EDTA (0.744g) were dissolved in distilled water (500ml) to give concentrations of 352mM, 48mM and 4mM, respectively. The solution was adjusted to pH 6.0 if necessary. Cysteine was added freshly as required before use (final concentration 0.968 mg/ml, ie 8mM).

Stopping buffer — Sodium acetate buffer, pH 4.3 (30mM sodium acetate, 70mM acetic acid) containing sodium chloroacetate (100mM).

Substrate — Z-Phe-Arg-AMC. This substrate was prepared as stock solution in DMSO (3mg/ml, 4.89mM) for storage (-20°C) and diluted in distilled water before addition to the assay, to give a concentration of 19.6µM (ie 1:250 dilution factor).

Reaction Product — 7 Amino-4-methylcoumarin (AMC) was prepared as a 50µM stock solution (8.76 mg/l). Gentle warming was necessary to dissolve the compound. Before use, it was diluted with distilled water to give a final concentration of 0.5µM.

All other reagents were as described in Chapter 2.

**Equipment**

| | |
|---|---|
| Fluorimeter | As described in Chapter 5. Zeroed using distilled water and full scale (1000 units or 100%) set using 0.5 µM AMC. The fluorimeter did not have a thermostatically-controlled sample chamber. |
| Chart Recorder | As described in Chapter 5. |

All other equipment as given in Chapter 2.

II. **METHODS**

8.2.1 Incubation of Yolk-Sac Tissue and Preparation for Assay

Yolk sacs were incubated in the presence and absence of leupeptin (100µg/ml), using the reduced volume method described in Section 2.2b. Incubation was generally over a period of 120min. At various periods, yolk sacs (duplicate tissue samples at each time point for both leupeptin-treated and control tissues) were removed from the incubation flasks, rinsed in three changes of ice-cold saline solution (1% w/v), and immediately frozen at -20°C. Yolk sacs that had not been incubated were also prepared for assay, by rinsing in saline and freezing at the beginning of the experiment.

After freezing yolk-sacs were homogenized in Triton X-100 (0.1% v/v; 1ml per yolk sac) using a hand-held glass-on-glass homogenizer. Tissue and homogenate were kept on ice whenever possible. The homogenates were then centrifuged (1000g, 15min) to remove cell debris, and the supernatant fraction used for assay of cathepsin B+L activity.

In some experiments, frozen, unincubated yolk sacs were homogenized in the presence of leupeptin (0.25-2.0µg) in order to assess the amount of leupeptin required to inhibit cathepsin B+L activity under the conditions of assay used. In other experiments fresh unincubated tissue was homogenized and assayed for enzyme activity immediately, with no storage at -20°C.

The protein content of the homogenate supernatant fraction was determined using the Folin method as described in Section 2.3., except that 20µl of the homogenate supernatant was added to 1.0ml of 0.5M NaOH.

8.2.2 Assay of Z-Phe-Arg-AMC Hydrolysing Activity

The assay procedure was based on that described by Barrett (1980). The assay was originally used for cathepsin B but later found to be more sensitive for cathepsin L (Barrett & Kirschke, 1981). The method suggested the use of a "stopped reaction" carried out at a temperature of 40°C over 10 minutes.

The method of Barrett (1980) was assessed for use with yolk-sac tissue. In particular, the amount of yolk-sac homogenate protein used was varied in an attempt to achieve readily-detectable effects. The method was adapted to a continuous rate assay, using a fluorimeter connected to a chart recorder. Thus for most experiments, the following assay procedure was used.

Reaction buffer (0.825ml, pre-warmed to 37°C in a water bath) and homogenate (50µl, stored on ice) were mixed in a cuvette, then substrate (0.125 ml, also pre-warmed to 37°C) added. The rate of ensuing hydrolysis was monitored, using the fluorimeter and chart recorder, for about 5 minutes or until 50% deflection was reached. The rate of reaction was measured in terms of fluorescence units per minute, then the enzyme activity calculated in units of ng AMC produced per minute per mg yolk-sac homogenate protein. Since 1000 florencence units, or full scale deflection, was set using 0.5µM AMC, one unit of fluorescence was produced by 0.0875ng AMC in the 1ml reaction volume. Hence activity ngAMC/min per mg yolk-sac protein could be calculated:-

$$\text{ng AMC/min/mg} = \frac{\text{slope of chart recorder trace (units/min)} \times 0.0875}{\text{mg protein in 50 µl homogenate}}$$

In some preliminary experiments a "stopped-reaction" assay was used. The method was similar to that described in detail by Barrett (1980). Briefly, a sample of centrifuged homogenate was diluted with

Triton X-100 (0.1%, v/v solution) by a factor of up to 1000. (This was necessary to achieve fluorescence readings within the set range). This diluted homogenate (0.25ml) was added to the reaction buffer (0.125ml) in a small reaction vial, then substrate (0.125ml) added to initiate the reaction. After a known reaction period at 37°C, (generally about 15 minutes) stopping buffer (0.5ml) was added. The fluorescence of the reaction mixture was then measured, after dilution if necessary.

## 8.3. RESULTS

Before reporting the results obtained it is pertinent to note some of the problems encountered with the method. As previously mentioned, several different assay conditions were tested in order to find a suitable method to use with experimental tissue. The factors that were varied included: amount of homogenate protein added; storage conditions of intact tissue and homogenate; reaction time; and concentration of substrate. Many of these variations caused large differences (up to 25-fold) in the apparent activity of cathepsin B+L, (nqAMC/min per mg homogenate protein). The results obtained during this investigation into optimal reaction conditions are given in Section 8.3.2.

The results obtained using experimentally-treated tissue are given in Section 8.3.1. In order to standardize these results, the same assay conditions were used for each experiment. To permit easier comparison of the experiments, the cathepsin B+L activity measured in yolk sacs that had been incubated with or without leupeptin was expressed as a percentage of that measured in the same experiment for unincubated tissue. In addition to interexperimental variability of the apparent enzyme activity, the enzyme activity measured in individual yolk sacs within a single experiment, treated and assayed in exactly the same way, differed greatly. This difference was particularly marked in unincubated tissue or tissue that had been incubated without leupeptin. In order to calculate the activity of incubated yolk sacs as a percentage of the activity measured in unincubated yolk sacs, it was necessary to use the mean enzyme activity of unincubated tissue; at least three yolk sacs were assayed to obtain this mean.

8.3.1. Effect of Incubating Yolk-Sac Tissue in the Presence and Absence of Leupeptin on the Residual Cathepsin B+L Activity

Yolk sacs were incubated (0-120 min) in medium with or without leupeptin (100 µg/ml). The cathepsin B+L activity of the homogenates of these yolk sacs was determined using the continuous rate assay described in Section 8.2.2., and expressed as a percentage of that measured in unincubated tissue from the same experiment. The results from two such experiments are shown in Figs. 8.1 and 8.2. (The experiment was repeated a total of five times, with similar results, not shown) There were several interesting features of these results.

i) The cathepsin B+L activity was greatly decreased in homogenates from yolk sacs that were incubated in the presence of leupeptin. After 120min exposure to leupeptin, the activity was lowered by 90-100%. The percentage of residual activity differed slightly between experiments, but reached a low, steady-state value in all experiments. The decrease in activity occurred very rapidly, the majority of activity being lost within the first 30min exposure to leupeptin; the inhibition of activity was maximal by about 60min.

ii) The cathepsin B+L activity measured in tissue incubated without leupeptin was markedly initially higher than that of unincubated tissue: the relative activity rose 200-400%. This apparent increase occurred within the first 10min of incubation. The activity then tended to decrease, after 120min incubation it was usually less than that observed in unincubated tissue.

iii) The figures illustrate the variability in cathepsin B+L activity between yolk sacs removed from incubation after the same period. The difference between some 'duplicate' samples was over 100%. Variability in the leupeptin-treated tissue was less, probably

because all activity was so strongly depressed.

Fig. 8.3 shows the cathepsin B+L activity in unincubated yolk sacs to which known amounts of leupeptin were added, expressed as a percentage of unincubated tissue to which no leupeptin was added. The activity decreased with increasing amounts of leupeptin and was virtually totally inhibited by 1.5µg of leupeptin per yolk sac. This suggests that only about 1.5µg of leupeptin would be required within an average yolk sac to achieve the almost complete inhibition of cathepsin B+L activity observed after about 60min incubation in the presence of leupeptin (100µg/ml).

### 8.3.2. Factors Affecting the Measurement of Cathepsin B+L activity in Unincubated Yolk Sacs

Preliminary investigations to determine suitable assay conditions were made using both the continuous assay and the stopped reaction methods. The effects of varying different factors on the calculated rate of reaction are discussed briefly below.

**8.3.2.a Stopped Reaction Method**

i) **Homogenate Protein Concentration** Duplicate yolk-sac homogenates were diluted between 10- to 1000-fold. These diluted homogenate fractions were assayed for cathepsin B+L activity using the stopped reaction method, and the rate of reaction expressed as ngAMC/min per mg yolk-sac protein. This calculated rate was strongly dependent on the amount of homogenate protein present. Surprisingly the rate increased non-linearly, as the amount of homogenate protein decreased (see Fig. 8.4). The rate of reaction (ngAMC/min) was similar for all concentrations of homogenate protein.

ii) **Substrate Concentration.** The concentration of Z-Phe-Arg-AMC in the stopped-reaction assay was varied between 6-9ng per ml. The

homogenate was used at dilutions of 1:200 and 1:500 for these experiments. At both homogenate dilutions the rate increased linearly with substrate concentration, (see Fig. 8.5) indicating that the substrate was not at a saturating concentration even for the more dilute enzyme concentration.

iii) **Reaction period.** The reaction period in the stopped reaction method was varied from 1 to 30min, to determine whether the time-course of reaction was linear. Homogenate supernatant was used at a dilution of 1:200, on the standard substrate concentration (12nq/ml) was added. Under these conditions, the rate of reaction was approximately constant over 30 min incubation at 37°C, (results not shown in detail).

iv) **Effect of storage of intact tissue on cathepsin B+L activity.** Homogenates were prepared from tissue that had been frozen at -20°C (for 6 days), and from freshly-dissected tissue. These freshly prepared homogenates were then assayed together for cathepsin B+L activity. Little difference in the activity of the homogenates was observed, suggesting that activity did not change on storage of intact tissue.

**8.3.2.b Continuous Rate Assay**

i) **Amount of homogenate protein.** Initially, the amounts of reagents used for the continuous rate assay were similar to those used for the stopped reaction, except the volume of reaction buffer was increased to compensate for the absence of stopping buffer. The yolk-sac homogenate was diluted up to 1:200 before addition. At 200-fold dilution, the reaction was too slow to be of use. At 1:5 dilution, the initial rate was approximately linear, and rapid enough to allow reasonably short assay periods. Additions of more homogenate to the reaction mix during assay gave a large increase in the rate of reaction.

ii) **Substrate Concentration.** No systematic investigation was made on the effect of changing the substrate concentration, but it was noted that the rate of reaction increased markedly when additional aliquots of substrate were added to the reaction mixture.

iii) **Effect of storage of tissue homogenate on cathepsin B+L activity.** Homogenates were prepared from frozen yolk-sac tissue, and the cathepsin B+L activity measured (as described in Section 8.2.2.). The homogenates were then stored at -20°C for up to 3 days, and re-assayed. The rate of reaction was slightly lower for the tissue homogenates that had been frozen. This could reflect a decrease in cathepsin B+L activity on storage, although day-to-day variation in the assay could also explain these results. For experimental tissue, all assays were carried out on the same day whenever possible, so that the activity compared to control, unincubated tissue was not affected by day-to-day differences in the assay. Since the rates were expressed as a percentage of a control value, the effect of storage on the 'absolute' activity of the enzymes were not as important, provided the change in activity was proportional in all tissues.

iv) **Temperature of Assay.** In the continuous rate assay, the initial temperature of the reactants was 37°C. However, the fluorimeter sample holder was unthermostatted (temperature about 25°C), so the temperature of the reaction mixture decreased during measurement of reaction rate. This caused a corresponding decrease in reaction rate. The curvature of the time-course of reaction depended on the initial rate of reaction; the higher the initial rate of reaction the more linear the time-course, since the period of cooling was less.

The initial approximately linear portion of the curve was used to measure the cathepsin B+L activity of experimental tissue.

Other factors that may have affected the assay were also checked. The fluorescence of AMC was directly proportional to its concentration over the range tested, and was not affected by the presence of protein (added after the stopping buffer) or Triton X-100. The presence of excess AMC in the assay reaction mixture only slightly inhibited the rate of reaction; the amount produced during the reaction was unlikely to do so.

## 8.4 DISCUSSION

### 8.4.1 Cathepsin B+L Activity in the Rat Yolk Sac

The results described in Section 8.3.1 are discussed below. It is important to realize that these results indicate only a general trend in the cathepsin B+L activity of incubated yolk-sacs. (Further investigations into optimum assay conditions and optimum methods of tissue preparation are necessary before reliable quantitative data can be reported.) Also, several assumptions must be made in order to interpret even the trends in the reported results. (These will be briefly discussed below.) Notwithstanding these reservations, the results suggested the following trends.

The cathepsin B+L activity decreased rapidly on incubation of the yolk-sac tissue with leupeptin (100 µg/ml). Generally only about 5% of the activity remained after approximately 60min incubation. This observation can be compared with results described in Chapter 3. In that chapter, $^{125}I\text{-}BSA_{fd}$ degradation was also maximally inhibited after 60min, but about 20% of the degradative capacity towards $^{125}I\text{-}BSA_{fd}$ remained beyond this point. The almost complete and rapid inhibition of cathepsin B+L observed in this chapter can also be contrasted with those observed by workers using leupeptin in other cells or tissues. Thus, Neff *et al.* (1979) detected only 25-50% inhibition of the cathepsin B activity of hepatocytes after 4h, and Tanaka *et al.* (1981) found that maximal inhibition, approximately 55%, occurred only after 10h. However, a stronger effect was detected by Kominami *et al.* (1980), who found that when leupeptin was administered to rats by intraperitoneal injection, maximal inhibition of cathepsin B in the lysosomal fraction of the liver occurred after 1.5h; 80% inhibition was achieved.

Thus it would appear that leupeptin induces a stronger, more rapid inhibition of cathepsin B+L in the rat yolk sac compared with

several other cell types incubated *in vitro*, though not necessary compared with inhibition of liver proteinases after injection of leupeptin *in vivo*.

The rapid onset of cathepsin inhibition in yolk sacs incubated with leupeptin can be compared with that induced by other inhibitors in various tissues. For example, Shaw & Dean (1980) found that Z-Phe-Ala-$CHN_2$ gave a 95% inhibition of cathepsin B activity in macrophages after 90min exposure. Hashida *et al.* (1982) found that the inhibitor E-64 (N-[N-(L-3-trans-carboxyoxiran-2-carbonyl) -L-leucyl]-agmatine) caused a maximal inhibition (about 75%) of cathepsin B 1h after intraperitoneal administration. Several workers have reported that pepstatin has little or no effect on proteolysis in intact cells (eg Knowles *et al.*, 1981; Tanaka *et al.*, 1981), however, Furuno *et al.* (1983) found that 1h after a pepstatin-asialofetuin complex was administered intraveneously to rats, the cathepsin D activity was inhibited by 80%. This difference in effect was ascribed to the rate of uptake of pepstatin complex (by receptor-mediated pinocytosis) being more rapid than that of free pepstatin (which is assumed to enter cells by fluid-phase pinocytosis). However, Z-Phe-Ala-$CHN_2$ and E-64 both gave rise to a similar time-course of inhibition, even though the former is thought to enter cells by pinocytosis and the latter by permeation. Hence it is not possible to distinguish between these possible modes of uptake of an inhibitor on the basis of the time-course of inhibition alone.

The apparent increase in cathepsin B+L activity of yolk sacs which occurred within minutes of incubation in the absence of leupeptin was unexpected and difficult to explain. Synthesis of new cathepsin is unlikely to have occurred within such a short period, and no changes in the proteolytic activity of yolk sacs during incubation have been observed previously (eg Knowles *et al.*, 1981).

[However, the rate-limiting step in the lysosomal degradation of endogenous and exogenous proteins is thought to be the delivery of the protein to the lysosomes (Williams et al., 1975b), therefore an increase in cathepsin activity would not automatically give rise to an increase in proteolytic rate.] The stimulation of cathepsin B+L observed in yolk sacs was an acute effect only, since the activity decreased to below that of unincubated tissue after 120 min. The cause of this decreased activity is also unknown.

The observed cathepsin B+L activity in a yolk-sac homogenate can only be related to that of the tissue in situ if three important assumptions can be made.

First, during the washing of the tissue all leupeptin must be removed from extracellular sites. Otherwise, on homogenization it would gain access to lysosomal enzymes and give rise to an overestimate of the amount of inhibition occurring in the intact tissue. (This problem was encountered by Shaw & Dean, 1980.) Three rinses in saline (1% w/v) were found to release $^{3}$H-leupeptin from the tissue surface (Section 6.4.5a) therefore it is reasonable to assume that any inhibition of cathepsin B+L observed was caused by internalized leupeptin.

Second, homogenization of the tissue could bring lysosomal proteases into contact with leupeptin from non-lysosomal intracellular sites (eg any present in the cytosol or pinosomes). Such access may be partially or totally prevented in intact tissue. Since it was impossible to quantitate the fraction of the total leupeptin in such compartments, it had to be assumed that the quantity of leupeptin in such compartments was negligible compared with the amount present within lysosomes.

Third, it was necessary to assume that any leupeptin that was bound to (and thus causing inhibition of) cathepsin B or L within

lysosomes *in situ*, remained bound during homogenization and assay. It is possible that the extensive dilution of the lysosomal contents (to obtain first a '1 yolk sac per ml' homogenate, and then further dilution of the homogenate in the assay reaction mixture) could cause dissociation of the enzyme and inhibitor. Moreover, any competition between enzyme substrate (Z-Phe-Arg-AMC) and inhibitor (leupeptin) would further confuse the interpretation of the observations. However, the high affinity of leupeptin for cathepsin B and L would tend to minimize any such dissociation. (Leupeptin has been reported to act as a pseudo-irreversible inhibitor of cathepsin L; Kirschke *et al.*, 1977).

It is not possible to compare directly the degree of inhibition observed on addition of leupeptin to unincubated yolk sacs during homogenization with that observed in yolk sacs that had been incubated with leupeptin, because of the apparent different in cathepsin B+L activity in incubated and unincubated tissue. However, it was possible to demonstrate that approximately 1.5 µg leupeptin is required to fully inhibit the cathepsin B+L activity of (unincubated) yolk sacs.

### 8.4.2. Problems Associated with the Method, and Possible Improvements

A number of problems were encountered during development of the assay method used in this chapter. Lack of time prevented a search for solutions to these problems, but, with hindsight, some could have been eliminated or reduced by small changes in the materials and methods. For example, the time-course of the reaction could have been made more linear if the cuvette-holder had been thermostatted to 37°C. Some of the variability between the rate of reaction on different days may have been reduced if the concentration of

substrate used had been increased to about 5 x $K_m$, since at this concentration the substrate is saturating, and small concentration differences resulting from error in preparing the substrate solution have no effect. (However, this would have produced an extremely rapid reaction rate, and would be prohibitively expensive). Variation both between individual yolk sacs and between experiments may also have been reduced by improving the method of preparing and storing yolk sacs. Some cathepsin B activity may have been lost during preparation at neutral pH, and ideally both cathepsin B and L should be stored at acid pH at -20°C. Cathepsin L retains its activity best at -20°C if EDTA and a thiol reagent are present, however cathepsin B loses activity more rapidly under these conditions (Kirschke et al., 1977). It was hoped that the presence of high concentrations of other proteins would stabilize the yolk-sac cathepsins during preparation and storage for these experiments.

The reason for the increase in reaction rate with increase in dilution of the homogenate was not fully investigated. It could have been caused by dissociation of endogenous inhibitors from cathepsin B + L at the higher dilution. Such inhibitors are known to exist in the cytosol, and would have access to the lysosomal cathepsins on homogenization.

In summary, although the data reported in this chapter are only limited and hence qualitative, and their interpretation is impossible without a number of assumptions, they suggest that:

i) The observed inhibition of cathepsin B+L is complete within 60 min incubation with leupeptin (100 µg/ml).

ii) The quantity of leupeptin required to fully inhibit the cathepsin contained in a yolk sac could be obtained by

fluid-phase pinocytosis within 60 min. [Pinocytosis occurs at a rate of about 3µl/mg/h, therefore a typical yolk sac would take up 15µl of extracellular fluid per hour. For leupeptin at a medium concentration of 100µg/ml, the amount taken up in 60min would be 1.5µg, which was sufficient to totally inhibit the cathepsin B+L activity of unincubated tissue.]

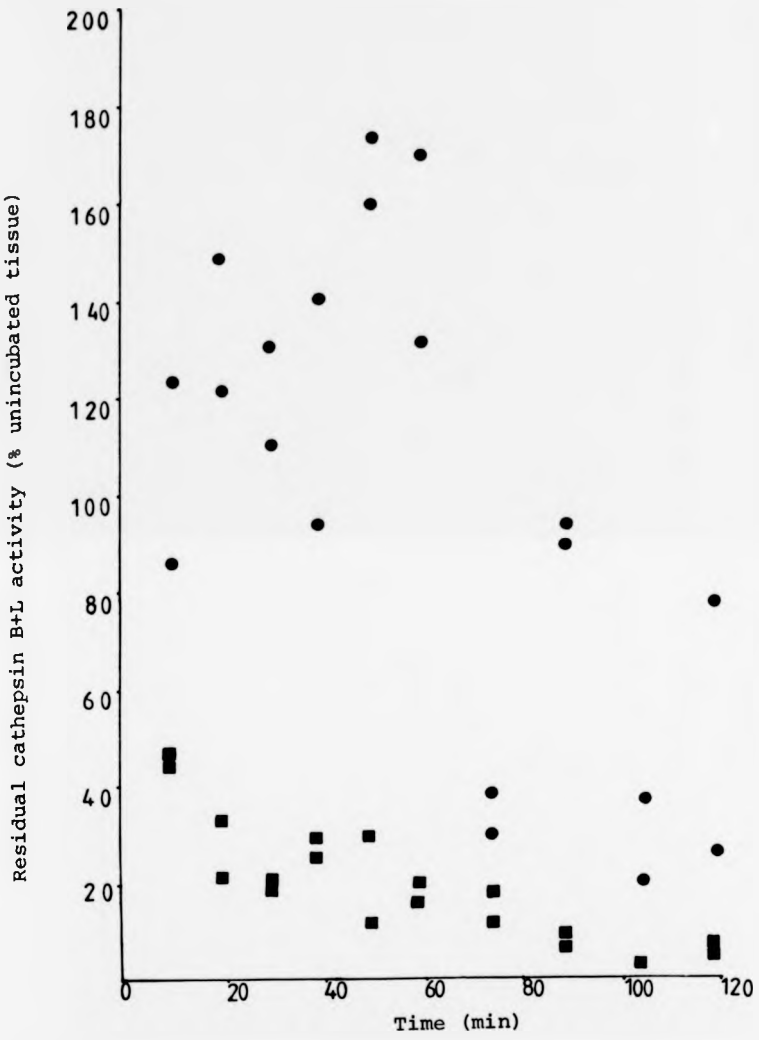



Fig. 8.1 Cathepsin B+L Activity of Yolk Sacs Incubated in the Presence and Absence of Leupeptin, Relative to Unincubated Tissue

Yolk sacs were incubated with or without leupeptin (100 µg/ml) as described in Section 8.3.1. The cathepsin B+L activity of this incubated tissue, and of unincubated tissue, was determined as described in Section 8.2.2 and the activity of incubated tissue expressed as a percentage of that of unincubated tissue. The graph shows results from a typical experiment.

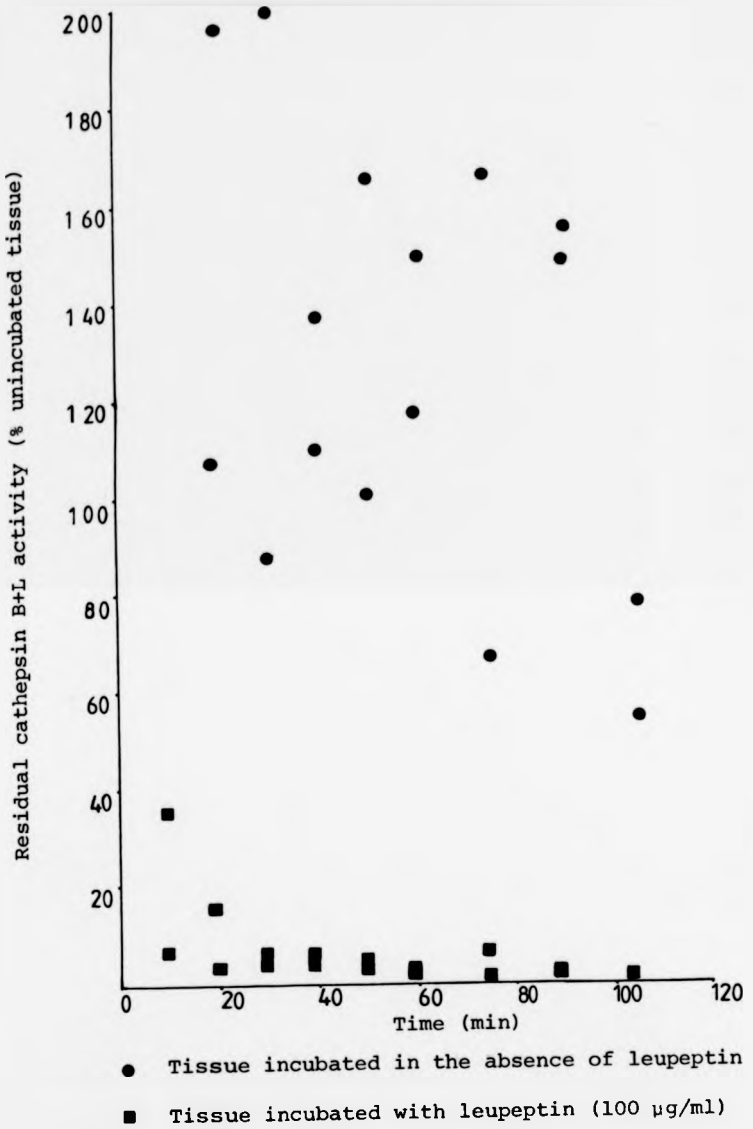



Fig. 8.2 Cathepsin B+L Activity of Yolk Sacs Incubated in the Presence and Absence of Leupeptin, Relative to Unincubated Tissue

Experimental details as described for Fig. 8.1. The graph shows results from a typical experiment

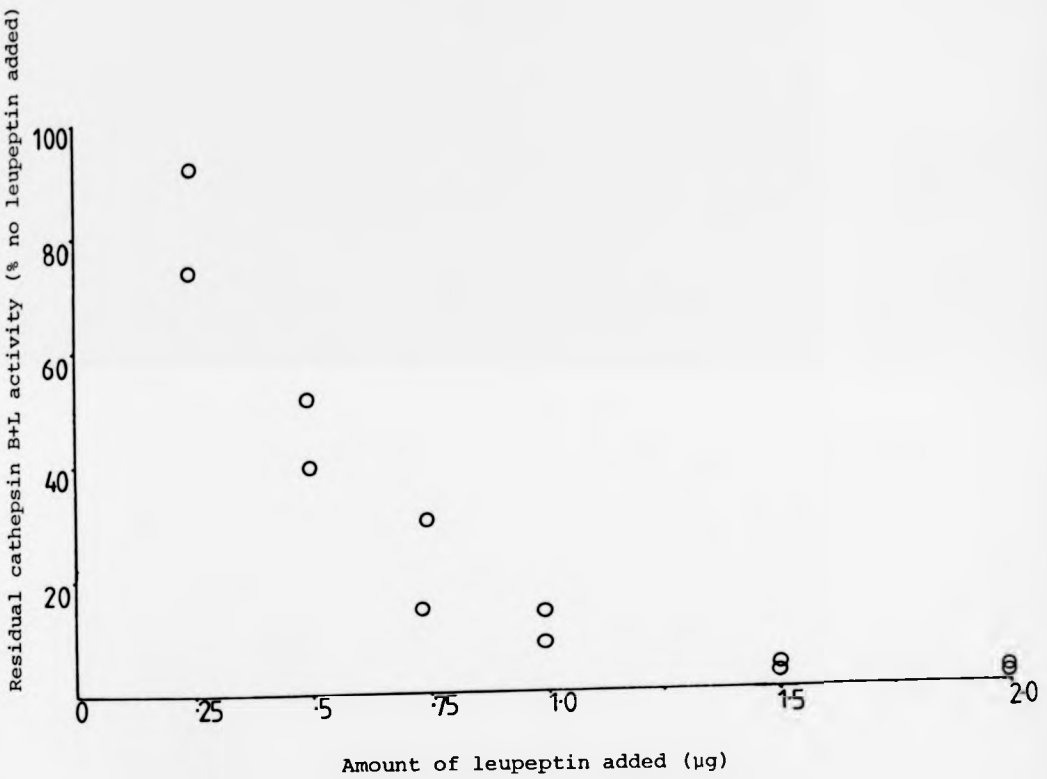



Fig. 8.3 Cathepsin B+L Activity of Yolk Sacs to which Leupeptin was Added

Known quantities of leupeptin was added to unincubated yolk sacs, which were then homogenized. The residual cathepsin B+L activity was measured and expressed as a percentage of that detected in unincubated tissue in the absence of leupeptin, as described in Section 8.2.1.

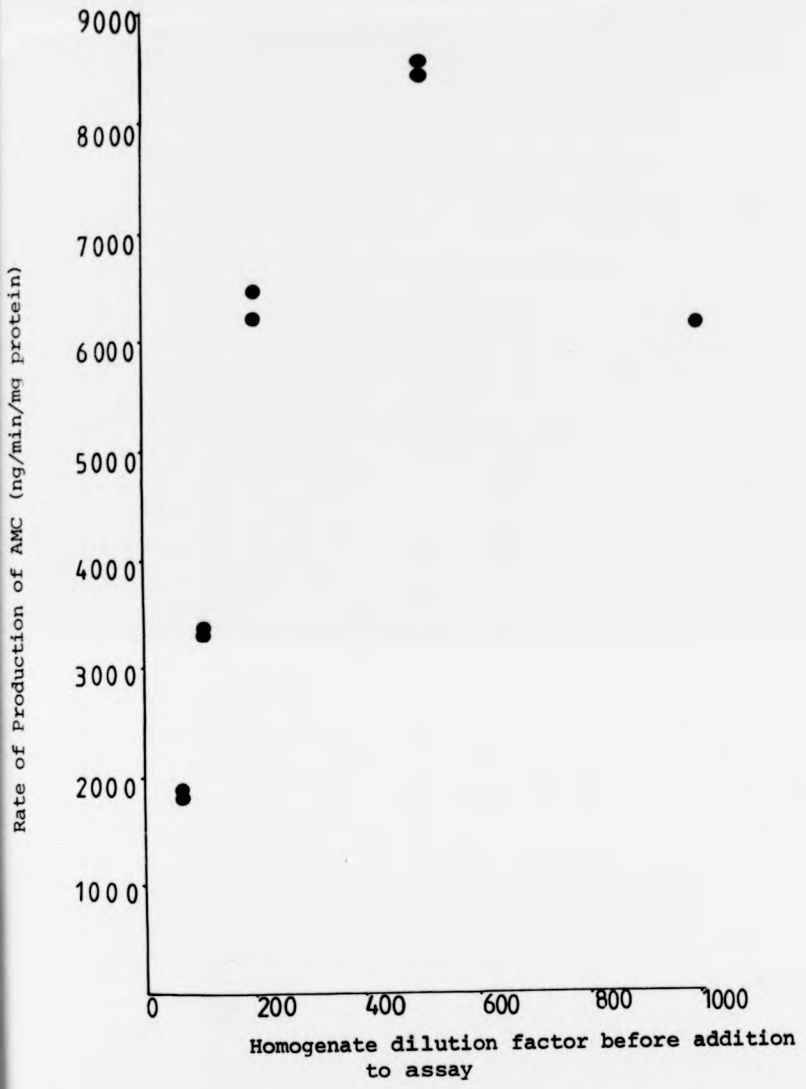
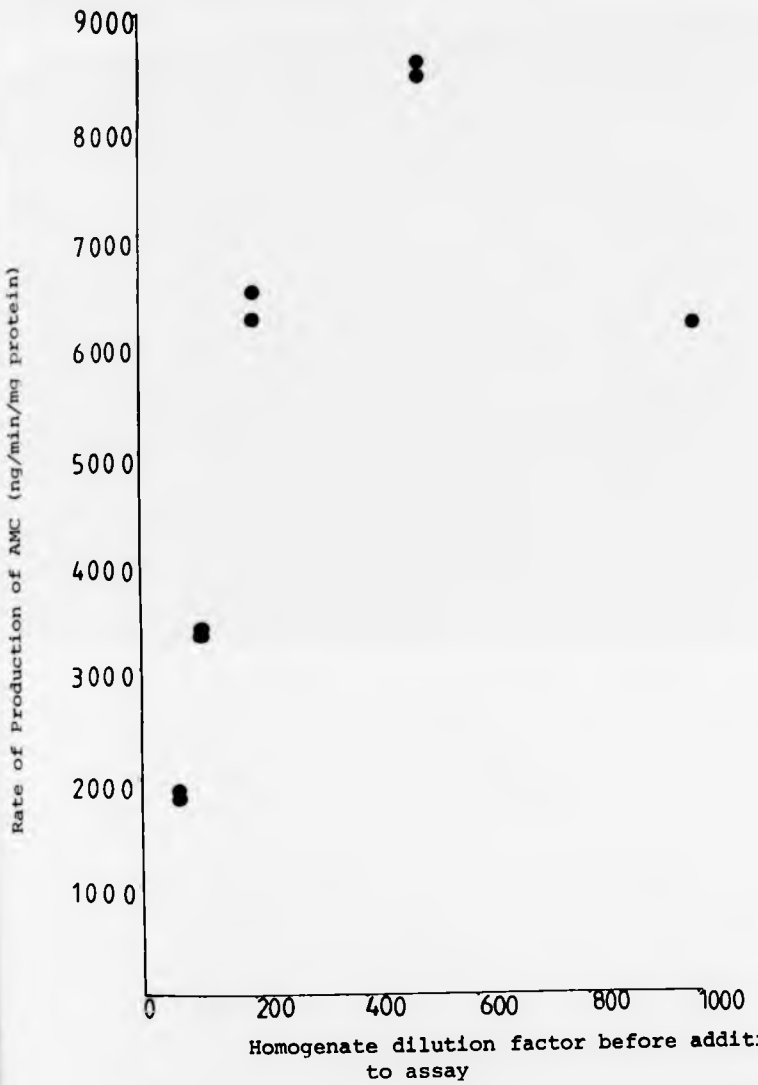



Fig. 8.4 Effect of Yolk Sac Homogenate Protein Concentration on the Measurement of Cathepsin B+L Activity by the Stopped Reaction Method

Unincubated yolk sacs were homogenized then the homogenate diluted up to 1000-fold in Triton X-100 (0.1% solution). The cathepsin B+L activity was determined as described in Section 8.2.2.

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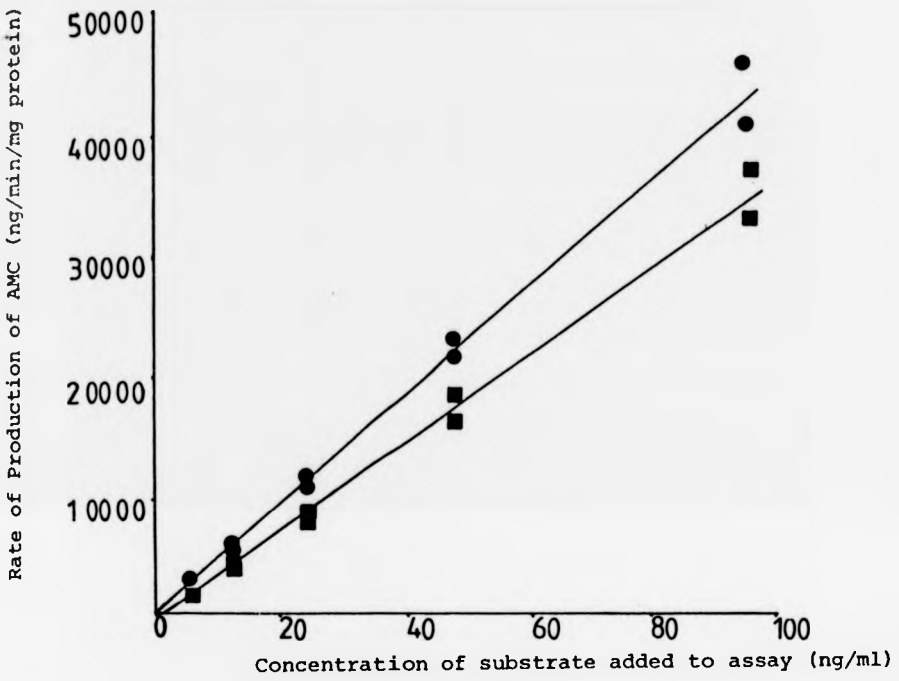



● Yolk sac homogenate diluted 1:500

■ Yolk sac homogenate diluted 1:200

Fig. 8.5 Effect of Substrate Concentration on the Rate of Production of AMC During the Stopped Reaction Cathepsin B+L Assay

The stopped reaction assay described in Section 8.2.2 was carried out using substrate at concentrations between 6 and 9 ng/ml with two concentrations of homogenate protein, as described in Section 8.3.2a

# CHAPTER 9

# GENERAL DISCUSSION

# GENERAL DISCUSSION

## 9.1 Aim of Work

The overall aim of the work reported in this thesis was to obtain more detailed knowledge of the mode of action of leupeptin as an inhibitor of proteolysis in intact mammalian cells and tissues.

The main problems encountered when using leupeptin as an inhibitor are the lack of information on:

i) The site(s) of action of leupeptin. [This depends on its mode of entry into cells and on the permeability features of intracellular membranes.]

ii) The degree of inhibition of proteolysis afforded by leupeptin. [This is dictated by the extent to which leupeptin-sensitive enzymes are involved in the rate-limiting steps of proteolysis in a given cell-type, and by the intracellular concentration of leupeptin (which depends on both the rate of entry of leupeptin into cells and on the possible rates of inactivation/degradation, or release of leupeptin).]

The first problem, ie that of the site(s) of action of leupeptin, has been noted by many workers. Tanaka *et al.* (1981) attempted to relate the effects of leupeptin (and pepstatin) on the turnover of endogenous cell proteins with inhibition of lysosomal proteolysis, but noted that "the possibility that the inhibitors may also act on proteinases in the cytosol to some extent is not completely excluded". Knowles *et al.* (1981) reported that leupeptin caused different degrees of inhibition in the degradation of endogenous and exogenous proteins in the rat yolk sac, and stated that "resolution of these findings requires information on the distribution and eventual intracellular fate of microbial proteinase inhibitors". Similar conclusions were reached by other investigators (Wildenthal, 1980; Henell & Glaumann, 1985; Ward *et al.*, 1979) using

other tissues and cells.

Dean and co-workers have also stressed the importance of knowing the site of action of inhibitors (eg Dean, 1979; Shaw & Dean, 1980; Cockle & Dean, 1982). These authors suggest that leupeptin probably enters cells by permeation (this conclusion being based on the rapid action of leupeptin; Dean, 1979) but state that "its site of action is unclear", Cockle & Dean (1982). The proteinase inhibitors pepstatin or Z-Phe-Arg-$CHN_2$ were used by Dean and co-workers because they were thought to enter cells by pinocytosis, and were thus assumed to be limited to inhibition of lysosomal proteolysis alone (Dean, 1979; Shaw & Dean, 1980).

The second problem, a lack of information on the degree of inhibition afforded by leupeptin, was noted by Henell & Glaumann (1985). They pointed out that, although many workers have assumed that leupeptin causes an almost complete cessation of lysosomal proteolysis, their results indicated that degradation of cell organelles within autophagolysosomes continued in the presence of leupeptin.

Hershko & Ciechanover (1982) sum up the use of inhibitors in a review of mechanisms of intracellular protein breakdown. Difficulties in assessing the amount of degradation occurring via lysosomes were discussed, and it was noted that "inhibitor studies are always subject to uncertainties concerning the specificity of the agent or completeness of its action".

Two models can be proposed to illustrate the possible action(s) of leupeptin in yolk sac. In one leupeptin is not able to permeate across the plasma membrane (Model A, Fig 9.1); in the other leupeptin is able freely to cross the plasma and possibly intracellular membrane(s) (Model B, Fig. 9.2).

In model A, the only route of uptake of leupeptin is by

fluid-phase or adsorptive pinocytosis. Pinocytic vesicles containing leupeptin fuse with lysosomes, resulting in delivery of leupeptin to lysosomes, and inhibition of lysosomal enzymes. If the lysosomal membrane is (like the plasma membrane) impermeable to leupeptin, the inhibitor will remain trapped within the vacuolar system. However, if the lysosomal (or pinocytic vesicle) membrane is permeable to leupeptin, leupeptin will be released into the cytosol, where it could inactivate non-lysosomal proteinases. Since pinocytosis is a constitutive process in the yolk sac, the rate of fluid-phase pinocytosis will be the minimum rate of uptake possible. (The rate of uptake via pinocytosis will be greater if leupeptin binds to the plasma membrane and is taken up by adsorptive pinocytosis.)

In model B, leupeptin is able to enter the cell by permeation across the plasma membrane, and is thus delivered to the cytosol. Uptake will also occur via pinocytosis, therefore the total rate of uptake by the cell will be the sum of the rates of pinocytosis and of permeation. The rate of accumulation of leupeptin by the tissue will not equal the total rate of uptake if loss of leupeption from the cells by permeation back across the plasma membrane occurs. Depending on the permeability of the lysosomal membrane towards leupeptin, the inhibitor will either permeate directly across the lysosomal membrane or be taken up by microautophagy and/or become enclosed within autophagic vacuoles (both processes are described by Dean, 1984).

Several different methods of detecting leupeptin were developed and utilized in the work described in this thesis. Each method provided evidence on different aspects of the action of leupeptin as an inhibitor of proteolysis in yolk-sac tissue. (The advantages, limitations and technical problems associated with each method and any possible mis-interpretation of results are discussed in detail in

the relevant experimental chapters, and will not be discussed further in this general discussion.) The combined results, giving an overall indication of the action of leupeptin in the rat visceral yolk sac, are discussed below.

Experiments were designed to investigate two main issues:-

1. **What is the mechanism of uptake of leupeptin into yolk-sac tissue?**

   Information on the mode of uptake of leupeptin would provide evidence on its initial location and site of action, as indicated by Models A and B.

2. **What happens to leupeptin once it is inside the cell?**

   Possible fates include:-

   i) Re-release of intact leupeptin from the cell by membrane permeation or, possibly, exocytosis.

   ii) Inactivation within the cell, in the cytosol and/or intralysosomally, for example by oxidation or reduction of the essential arginal group.

   iii) Degradation within the cytosol and/or lysosomes, to yield products (amino acids) that may be re-utilized by the cell or may permeate freely out of the cell.

   The question of what happens to intracellular leupeptin also covers the problem of which intracellular location(s) are accessible to leupeptin. This depends on the mode(s) of uptake and on the permeability of intracellular membranes towards leupeptin.

   The interpretation of the results is discussed below.

## 9.2 Mechanism of Uptake

The mechanism of uptake of leupeptin was investigated by monitoring the rate of accumulation of leupeptin, the effect of inhibitors of pinocytosis on the accumulation of leupeptin, and the saturability of accumulation. These methods have been used by others to distinguish between adsorptive and fluid-phase pinocytosis (eg Pratten *et al.*, 1980; Silverstein *et al.*, 1977). Similar methods, eg saturability, competition from substrate analoques, and determination of the rate of uptake, have been used to characterise membrane transport systems (Matthews & Burston, 1983).

Some of these methods have been used in the past to characterise the uptake of other peptides. For example, the peptide proteinase inhibitor Z-Phe-Arg-$CHN_2$ entered cells at about the same rate as markers of fluid-phase pinocytosis, therefore uptake was assumed to occur by this mechanism (Shaw & Dean, 1980). Uptake of $(D\text{-}Ala)_3$ and of $(D\text{-}Glu)_2$ was thought to occur via pinocytosis because their entry into cells was inhibited by the metabolic inhibitors of pinocytosis, parafluorophenylalanine and 2,4,-dinitrophenol (Ehrenreich & Cohn, 1969).]

The main leupeptin-detection methods used to study the mechanism of uptake of leupeptin were the fluoroqenic enzyme assay (in which only active, inhibitory leupeptin was detected), and $^{3}H$-monitoring (in which active and any inactivated leupeptin, degradation products of leupeptin, or cell components containing re-utilized label, were detected.) The $^{125}I\text{-}BSA_{fd}$ probe method was also used to suggest the mechanism of uptake of leupeptin.

The $^{3}H$-monitoring method indicated that accumulation of leupeptin did not become saturated over the concentration ranqe 3-100 µg/ml. The only mechanisms of uptake that are truly non-saturable are fluid-phase pinocytosis and non-mediated passive diffusion,

suggesting that leupeptin uptake may occur via one of these mechanisms. [However, if a saturable transport mechanism (carrier-mediated transport or adsorptive pinocytosis) involves a transport molecule with a low affinity for leupeptin, saturation might not be observed over the limited concentration range tested, thus saturable transport mechanisms cannot be entirely ruled-out from the available results.]

Both the $^{3}H$-monitoring and the fluorogenic enzyme assay results indicated that, in yolk-sac tissue, the rate of accumulation of leupeptin was similar to that of markers of fluid-phase pinocytosis (at least over the first 3h of incubation). This rate was the minimum expected, since uptake of leupeptin by permeation or by adsorptive pinocytosis would increase the rate of leupeptin uptake relative to that for fluid-phase pinocytosis. Hence the observed rate of accumulation of leupeptin suggests that the main mechanism of uptake is by fluid-phase pinocytosis.

However, the methods used only monitor the net rate of tissue accumulation of leupeptin, not the total rate of uptake. The rate of uptake will exceed the rate of accumulation if loss of leupeptin occurs, either by inactivation (detected by the fluorogenic assay) and/or by the release of intact of degraded leupeptin (detected by both the fluorogenic and the $^{3}H$-monitoring assay). Thus the true rate of uptake may be greater than that indicated by using either of the above methods.

The mechanism of uptake was further investigated using inhibitors of pinocytosis, in order to determine the proportion of leupeptin accumulation that could be blocked. The radiotracer assay indicated that the pinocytic inhibitors rotenone ($10^{-5}M$) and low temperatures (4-34°C) prevented association of leupeptin with yolk-sac tissue. The fluorogenic assay method produced similar

results, indicating that ammonium chloride (20mM) and low temperature (4°C) are effective inhibitors of leupeptin accumulation. Similarly, the $^{125}I$-$BSA_{fd}$-probe method suggested that entry of leupeptin into yolk-sac lysosomes could be partially blocked by ammonium chloride and by low temperatures.

However, the inhibitors of pinocytosis used may have affected uptake processes other than pinocytosis. For example, low temperature can also inhibit permeation across membranes (Fox, 1975). Alternatively, these inhibitors may have induced a more rapid loss of leupeptin from the tissue (eg ammonium chloride was shown to enhance the loss of leupeptin activity from yolk sacs, as detected by the fluorogenic assay, Section 5.4.2). It is unlikely, however, that all three inhibitors tested would each inhibit an alternative route of leupeptin uptake to the same extent, or that, for each inhibitor, the degree of inhibition of the alternative route would be the same as that achieved for pinocytosis. It is also unlikely that all three inhibitors caused a rapid loss of leupeptin from tissue. (Even ammonium chloride, which enhanced the loss of active leupeptin, did not increase the release of leupeptin-derived $^{3}H$-label from the tissue, thus could not have produced a rapid loss of $^{3}H$-leupeptin following uptake.)

Considered together, the observations of non-saturability, the rate of accumulation being similar to the rate of fluid-phase pinocytosis, and tissue-accumulation being inhibited by known inhibitors of yolk-sac pinocytosis, all suggest that the major mode of uptake of leupeptin is via fluid-phase pinocytosis.

## 9.3 Intracellular Fate of Leupeptin

Loss of leupeptin could occur by one or more of the following mechanisms: re-release of intact leupeptin, inactivation (possibly followed by release of inactivated leupeptin), or degradation followed by release of the degradation products.

Three leupeptin assay methods were used to investigate loss of leupeptin. The fluorogenic enzyme assay was used to monitor loss of active leupeptin from loaded yolk sacs. (The method was sensitive to small amounts of leupeptin, and distinguished between active and inactive forms.) Liquid scintillation counting was used to monitor release of $^{3}H$-labelled material from loaded yolk sacs into the re-incubation medium. (Although more sensitive than the fluorogenic assay, time did not permit a distinction to be made between active, inactive and degraded leupeptin by the $^{3}H$-monitoring method.) The chromogenic enzyme assay was used to characterise inactivation of leupeptin by a yolk-sac homogenate, and to monitor any inactivation of leupeptin in the medium in the presence and absence of yolk-sac tissue.

Results from the chromogenic assay method suggest that little or no inactivation of leupeptin occurred in the medium during incubation of yolk-sac tissue. However, inactivation of leupeptin was mediated by enzyme(s) present in a yolk-sac homogenate, but as the site(s) of inactivation were not determined, it was not known if inactivation could occur in the intact tissue.

An indirect indication that leupeptin activity was lost from intact tissue was given by monitoring uptake of leupeptin via the fluorogenic assay. The activity of intracellular leupeptin reached a steady state after 3h, when the rate of loss equalled the rate of uptake. This observation did not, in itself, allow distinction between the possible mechanisms of loss. However, no steady-state

was observed when monitoring $^{3}H$-labelled leupeptin uptake via $^{3}H$-monitoring. This suggests that loss of leupeptin was not caused by release of leupeptin (either intact, inactivated, or degraded) from the tissue, since such a release would have given rise to a steady-state quantity of $^{3}H$-label in the tissue.

Loss of leupeptin was further investigated by re-incubating leupeptin-loaded yolk sacs in leupeptin-free medium. Results using the fluorogenic assay indicated that 90% of leupeptin activity was lost from the tissue within 3h of re-incubation. Such loss was greatly enhanced by ammonium chloride (10mM). The rate of loss of leupeptin decreased during the re-incubation period, reaching zero after 3h in the absence, or 1h in the presence, of ammonium chloride. Results using the $^{3}H$-monitoring method were very different from equivalent results obtained using the fluorogenic assay. Only 28% of the $^{3}H$-label was released into the re-incubation medium over a 4h period, and ammonium chloride had no effect on this process. Moreover, the rate of release was approximately constant over the re-incubation period between 1-3h.

The disparity between the results from fluorogenic and $^{3}H$-monitoring methods again suggests that loss of leupeptin activity from the tissue was not entirely caused by release of intact leupeptin from the yolk sacs. (This conclusion is also supported by the lack of detection of active leupeptin released into the re-incubation medium by the fluorogenic assay.) Either leupeptin was inactivated by yolk-sac tissue and the inactivation product(s) retained within the tissue, or membrane-permeable $^{3}H$-labelled leupeptin degradation products were very rapidly re-utilized within the tissue, so preventing their release.

Ammonium chloride and other weak bases can affect cells in many ways (Dean, 1984; Seglen, 1983) thus their effects cannot immediately

be attributed to any one action. Several possible actions could each explain the increased loss of leupeptin observed using the fluorogenic enzyme assay (Section 5.4.2). Some of these were subsequently ruled-out by results from the radiometric assay and from the lysosomal permeability investigation. Release of $^{3}$H-label from $^{3}$H-leupeptin loaded yolk sacs was not enhanced by the presence of ammonium chloride. This indicates that neither total degradation of leupeptin (with attendant release of membrane-permeable degradation products) nor any mechanism of release of active/inactivated leupeptin was stimulated by ammonium chloride. Ammonium chloride did not affect the release of non-degradable pinocytic markers from yolk sacs, which suggests that plasma-membrane permeability, cell lysis and the rate of exocytosis were not radically affected, therefore these mechanisms cannot account for the increased loss of leupeptin. The lysosomal membrane of liver cells was observed to be freely permeable towards leupeptin (Section 7.4.2) therefore ammonium chloride is unlikely to act by causing an increase in the permeability of lysosomal membranes of yolk sacs so allowing inactivation of leupeptin by enzymes to which it was not normally exposed.

The most likely explanation is therefore that the increase in lysosomal pH in the presence of ammonium chloride gave rise to an increase in the rate of inactivation of leupeptin, which implies that the lysosomal system must be at least partially responsible for such inactivation. Stimulation of inactivation by raising the pH of the lysosomes with a lysosomotropic amine was not expected, and suggests that lysosomal enzyme(s) with a relatively high pH optimum were involved (as suggested in Section 5.4.2.)

Because loss of leupeptin from tissue clearly occurred, it is probable that the observed rate of accumulation of leupeptin (as

monitored by both the fluorogenic and $^{3}H$-monitoring methods) was an underestimate of the total rate of uptake.

Although 90% of leupeptin activity (as detected by the fluorogenic assay) was lost within 3h of incubation in leupeptin-free medium, the results of the $^{125}I\text{-}BSA_{fd}$-probe assay indicate that those leupeptin-sensitive lysosomal enzymes responsible for $^{125}I\text{-}BSA_{fd}$ degradation remained maximally inhibited after 5.5h. This could suggest that the 10% of active leupeptin (approximately equal to 0.08 µg/mg yolk-sac protein) that remained in the tissue after 3h was sufficient to maximally inhibit such enzymes. However this suggestion is not supported by the results of the cathepsin B+L assay, in which it was noted that total inhibition of the cathepsins B and L in a homogenate of a single yolk sac was achieved by the addition of at least 1.5 µg leupeptin. This is equivalent to about 0.3 µg leupeptin per mg yolk-sac protein and is thus much greater than the amount remaining within yolk sacs after a 3h re-incubation period.

The investigation of the intracellular fate of leupeptin included a study of the permeability of the lysosomal membrane towards leupeptin, in order to suggest which intracellular location(s) were available to leupeptin. A method first developed by Mego & McQueen (1965a,b) was used. Lysosomes isolated from rat liver rather than from yolk-sac tissue were used for these experiments. It was shown that leupeptin could enter isolated lysosomes freely, but no information could be obtained on the movement of leupeptin out of lysosomes. [Although it is theoretically possible to load liver lysosomes with $^{3}H$-labelled leupeptin *in vivo*, such experiments would be prohibitively expensive to conduct.] If the yolk-sac lysosomal membrane is freely permeable towards leupeptin, both entry into and exit of leupeptin from the lysosome may occur readily, leading to

leupeptin having access to both lysosomal and non-lysosomal compartments of the cell whatever the uptake mechanism by the cell.

## 9.4 Effectiveness of Leupeptin as an Inhibitor of Proteolysis in Intact Cells

The degree of inhibition of proteinase enzymes brought about by various concentrations of leupeptin after various periods of exposure was investigated to determine both the maximum degree of inhibition possible in intact yolk-sac tissue, and the incubation conditions necessary to achieve this maximum. The methods used for these investigations were the $^{125}$I-BSA$_{fd}$-probe, and the cathepsin B+L assay.

The $^{125}$I-BSA$_{fd}$-probe method clearly demonstrated that the degree of inhibition of leupeptin-sensitive enzymes reponsible for $^{125}$I-BSA$_{fd}$ degradation depended on both the concentration of leupeptin in the incubation medium and the period of exposure to the inhibitor. Maximal inhibition of $^{125}$I-BSA$_{fd}$ degradation was not achieved until after 1h of exposure to leupeptin at 100 µg/ml or 3h for leupeptin at 50 µg/ml. [Leupeptin at 25 µg/ml did not induce maximal inhibition even after a 3h exposure period.] These results concur with those of the cathepsin B+L assay for which maximal inhibition of cathepsin B+L was observed after about 30-60 min incubation with leupeptin at 100 µg/ml.

Using the $^{125}$I-BSA$_{fd}$ probe method, the maximum degree of inhibition observed was 80%. This incomplete inhibition of $^{125}$I-BSA$_{fd}$ degradation could have arisen either because leupeptin-sensitive enzymes were not totally inhibited by the maximum concentration of leupeptin achievable in the yolk sac, or because 20% of $^{125}$I-BSA$_{fd}$ degradation occurred via leupeptin-insensitive enzymes. These two possibilities could not be differentiated by the

$^{125}$I-BSA$_{fd}$-probe assay. However, results from the cathepsin B+L assay indicated that cathepsins B and L (which are the major lysosomal proteinases sensitive to leupeptin) were totally inhibited after incubation of yolk sacs with leupeptin. Thus the small amount of degradation of $^{125}$I-BSA$_{fd}$ that continued after incubation with leupeptin was probably mediated by leupeptin-insensitive enzymes. The alternative explanation (ie that a maximum steady-state concentration of leupeptin, capable of inducing an 80% inhibition of $^{125}$I-BSA$_{fd}$ degradation, occurred after 1h exposure to leupeptin at 100 µg/ml) could be rejected on the basis of results in which uptake of leupeptin was monitored using the fluorogenic assay. A steady-state concentration of active leupeptin was only observed after 3h incubation with leupeptin at 100 µg/ml; the amount of leupeptin in the tissue increased over 1-3h even though the degree of inhibition of $^{125}$I-BSA$_{fd}$ degradation remained constant.

These results illustrate the importance of an adequate pre-incubation period of yolk-sac tissue with leupeptin in order to achieve full inhibition of leupeptin-sensitive proteinases. The duration of a suitable pre-incubation period would depend on the concentration of leupeptin used. The results also demonstrate that the major leupeptin-sensitive lysosomal cathepsins, B and L, can be fully inhibited by incubating intact tissue with leupeptin, but such inhibition may not give rise to total inhibition of lysosomal proteolysis. Even though loss of leupeptin activity from the yolk sac was shown to occur by the fluorogenic assay, the steady-state concentration of leupeptin in the tissue was sufficient to induce maximal inhibition of the enzymes responsible for $^{125}$I-BSA$_{fd}$ degradation, and/or cathepsins B+L.

## 9.5 Comparison of Results with those in the Literature

Several reports of experiments with leupeptin have postulated possible modes of uptake of leupeptin, although the evidence on which such suggestions are made is often challengable. Both pinocytosis and permeation have been proposed as the route of entry of leupeptin.

The most frequently suggested mode of leupeptin uptake is membrane permeation. The main argument for this is the observed rapidity of action of leupeptin. [Unlike results observed in the yolk sac, in many cells and tissues there is little or no delay in the onset of leupeptin-induced inhibition. For example, Seglen *et al.* (1979), and Grinde & Seglen (1980), indicated that onset of inhibition of protein degradation was immediate on addition of leupeptin to hepatocytes cultured *in vitro*.] This rapidity of action may be contrasted with that of the peptide proteinase inhibitor pepstatin, which takes several hours to achieve inhibition of proteolysis in macrophages (Dean, 1979). Dean therefore proposed that leupeptin uptake is by membrane permeation, whereas pepstatin enters cells by pinocytosis alone (Dean, 1979). However, other explanations are possible. For example, leupeptin-sensitive enzymes may catalyse the rate-limiting steps of proteolysis, or be responsible for a greater proportion of proteolysis, so that inhibition by leupeptin is more readily detectable than that by pepstatin. Alternatively, leupeptin may inhibit its target enzymes more effectively at a lower concentration than pepstatin. It should also be noted that several reports suggest that onset of inhibition by leupeptin is not immediate. For example, Tanaka *et al.* (1979) reported that maximal inhibition of cathepsin B-type activity in rat-liver cells did not occur until after 10h exposure to leupeptin (50 µg/ml), and Sher *et al.* (1981) reported that $Ca^{2+}$-activated proteinase activity of muscle was maximally inhibited 24-48h after

intraperitoneal administration *in vivo*.

Another indication for a non-pinocytic route of entry for leupeptin was made by Cockle & Dean (1982), who noted that leupeptin produced similar effects in Swiss 3T3 and human fibroblast cells, but pepstatin and Z-Phe-Arg-$CHN_2$ (both of which were thought to enter cells by pinocytosis) produced a greater effect on human fibroblasts than on 3T3 cells. This difference in degree of inhibition was proposed to be caused by differences in the rates of pinocytosis in the two cell types, the effect of leupeptin being similar in both types because of its non-pinocytic mode of entry. [Again, other explanations are possible, for example smaller amounts of leupeptin may be required to produce a maximal effect in both cell types, so the rate of pinocytosis in each type may be unimportant. Moreover, it has been noted by Pratten *et al.* (1980) that the rates of pinocytosis in 3T3 and normal human fibroblasts are very similar.]

Seglen (1983) noted that intact leupeptin was rapidly transferred from liver sinusoids to the bile and suggested that leupeptin was thus readily transported across cell membranes. However, alternative rapid routes of entry into the bile exist, such as paracellular diffusion across cell junctions and diacytosis within vesicles (Lowe *et al.*, 1985).

A suggestion that uptake of leupeptin may occur via permeation was made by Borin *et al.* (1981), who stated that leupeptin may be useful as an antifertility agent because it was able to permeate, by fast transmembrane diffusion, the acrosomal membranes of intact spermatozoa and inhibit intracellular acrosin. However, Schill *et al.* (1981) reported evidence that leupeptin was not able to inhibit acrosin within intact spermatozoa.

To date, the only indication that leupeptin may enter cells by pinocytosis was suggested by Neff *et al.* (1979) who found that

leupeptin did not inhibit proteolysis in intact hepatocyte lysosomes. They therefore proposed that leupeptin gained entry to lysosomes via a pinocytic mode of uptake or via autophagy.

In view of the growing acceptance in the literature of the opinion that leupeptin enters cells by permeation (eg Cockle & Dean, 1984; Borin et al. 1981), it was somewhat suprising to find that, in yolk-sac tissue, the experimental evidence suggests that the major route of uptake of leupeptin was via fluid-phase pinocytosis. It is quite possible, however, that in cells that are less pinocytically active than yolk-sac epithelial cells (eg muscle), entry by permeation would provide a major contribution to uptake. It is also possible that the permeability of the plasma membranes of different cell types towards leupeptin may differ (West, 1983).

None of the reports that suggest uptake of leupeptin occurs via membrane permeation have characterised the mechanism of permeation (ie non-mediated, facilitated, or active transport). The size and polar nature of leupeptin would be expected to exclude entry by passive diffusion across the phospholipid bilayer, though possibly the molecule would enter via 'aqueous pores' in the membrane (Matthews, 1977), or via protein pores or channels (Alberts et al., 1983). Non-substrate-specific active transport systems for di- and tripeptides are known to exist in the gut. However, those characterised to date would not be expected to have any affinity for leupeptin (Matthews, 1977). The presence of such general transport mechanisms in tissues other than the intestine has not been established. Many biologically-active peptides are known to occur in mammals some of these have specific transport mechanisms in particular tissues to allow their entry and/or exit. Several such systems are discussed by Matthews & Payne (1975). Although there is no reason for a transport mechanism specific for leupeptin to be

present in mammalian membranes, it is just possible that leupeptin may utilize a system that normally transports other biologically-active peptides. Discussions of peptide transport systems are given by Matthews and Payne (1975) and in the Ciba Foundation Symposia (1972 and 1977).

The intracellular fate of leupeptin has also been discussed by several workers. Many reports suggest that leupeptin may be inactivated *in vivo* and *in vitro* (see Chapter 6 for discussion of such reports). The most convincing evidence for leupeptin inactivation is provided by Beynon & co-workers, who demonstrated leupeptin inactivation by homogenates of many different tissues, and who isolated an enzyme from rat liver capable of cleaving the Leu-arginal bond of leupeptin. Further evidence of leupeptin degradation was reported by Tanaka *et al.* (1983) who isolated metabolites of leupeptin after intravenous administration to rats. Acetyl-Leu-Leu was identified as one such metabolite. The results reported in this thesis do not contradict the published data, in that leupeptin inactivation also appears to have occurred in the yolk sac. However, the indication that leupeptin inactivation products were not released from yolk-sac tissue does not concur easily with the reported cleavage of leupeptin to form Ac-Leu-Leu and arginal. Only if Ac-Leu-Leu cannot be further broken down and if arginal cannot utilize amino acid membrane tranpsort systems would these products be expected to be retained within the yolk sac, unless rapid metabolism and/or re-utilization occurred.

## Future Work

Had time permitted, the experiments described in this thesis could have been extended in several different directions. The possibility of uptake by permeation could have been investigated

further by using a wider range of leupeptin concentrations and/or by using leupeptin analogues as competitive inhibitors of mediated transport. It would also have been interesting to monitor the effect of ammonium chloride and low temperature on leupeptin uptake using the cathepsin B+L assay, to determine whether cathepsins B and L could be inhibited by any leupeptin entering the tissue in the presence of these pinocytic inhibitors. The results could then have been compared with the inhibition studies carried out using other leupeptin detection methods. (However, the cathepsin B+L assay method itself would require further modification to obtain more reproducible results before future use.)

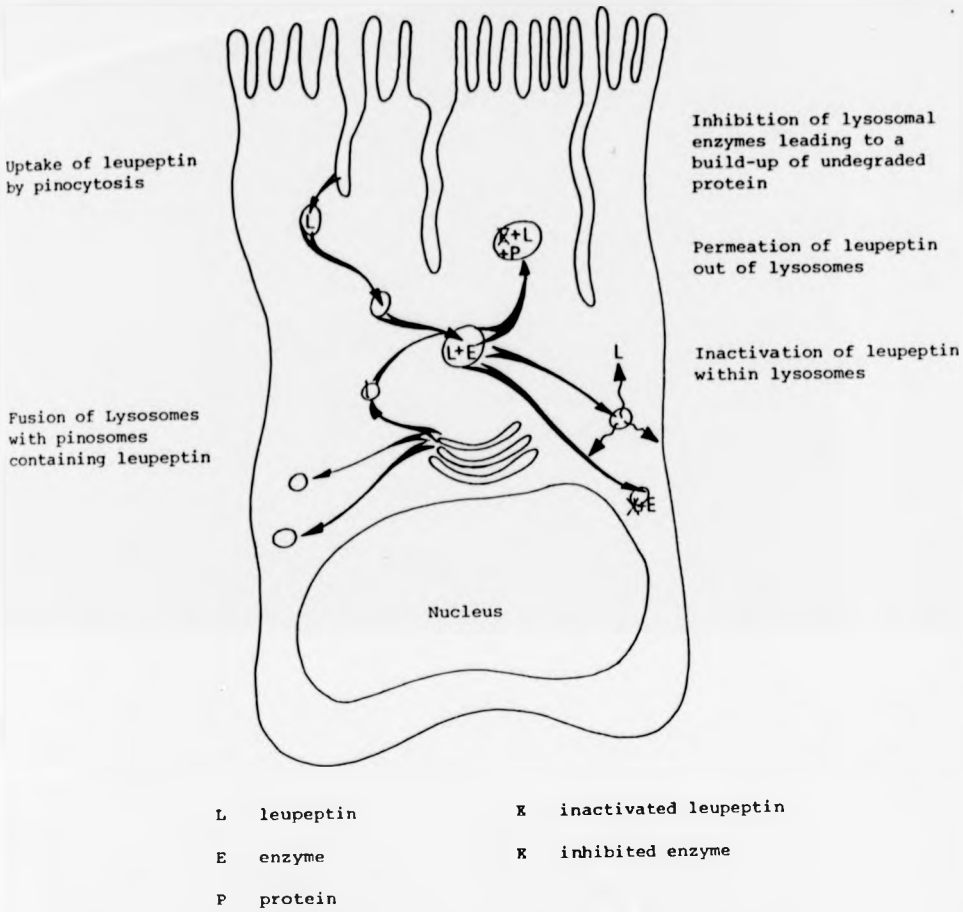
Many features of the intracellular fate of leupeptin in yolk-sac tissue remain unresolved. Future work could include an investigation of the leupeptin metabolites produced in the yolk sac, in order to determine why leupeptin inactivation did not lead to release of degradation products. Such an investigation would require methods for separating, detecting and identifying any such metabolites (some of the methods tested for use are described in Appendix 4). It would also be interesting to further investigate the location and characteristics of enzymes involved in leupeptin inactivation/degradation. For example, the inactivation of leupeptin by isolated lysosomal and cytosolic enzymes could be measured. (Crude preparations of such enzymes could be obtained from rat liver tritosomes and from a particle-free supernatant of a liver homogenate.) Results from such experiments may give a better indication of the site of inactivation/degradation of leupeptin. Also the pH dependence of inactivation/degradation by a lysosomal extract could be measured (using the sensitive fluorogenic assay) to indicate whether inactivation occurs more rapidly at neutral pH.

In conclusion the results of these investigations show that

fluid phase pinocytosis is a major route of uptake of leupeptin in rat yolk-sac tissue, but the possibility of additional mechanisms of uptake cannot be ruled out entirely.

Leupeptin appears to be inactivated within yolk sacs, however it achieves an intracellular concentration great enough to fully inhibit cathepsins B+L and remains sufficiently active to inhibit protein degradation 5h after exposure of tissue to inhibitor.

The intracellular location of leupeptin was not fully established. It clearly has access to lysosomes via its pinocytic route of uptake, and can, *in vitro*, permeate into the lysosome across the lysosomal membrane. However the likelihood of leupeptin permeating into the cytosol, either from lysosomes or from the extracellular medium, was not determined. Thus it is not possible to assess whether leupeptin inhibits only intralysosomal protein degradation or whether it is also capable of inhibiting non-lysosomal proteinases *in vivo*. Investigations into these factors are worth persuing further, in order to establish whether leupeptin can be used to determine the importance of lysosomal and non-lysosomal pathways of proteolysis in cells.




L leupeptin

E enzyme

P protein

K inactivated leupeptin

K inhibited enzyme

Fig. 9.1 Possible Intracellular Fate of Leupeptin MODEL A: Plasma Membrane Impermeable Towards Leupeptin

Uptake of leupeptin would occur via pinocytosis, leading to delivery of leupeptin to lysosomes. Leupeptin would then inhibit lysosomal enzymes and may become inactivated within lysosomes. It is also possible that intact leupeptin may permeate out of lysosomes to inhibit cytosolic proteinases (or become inactivated itself).

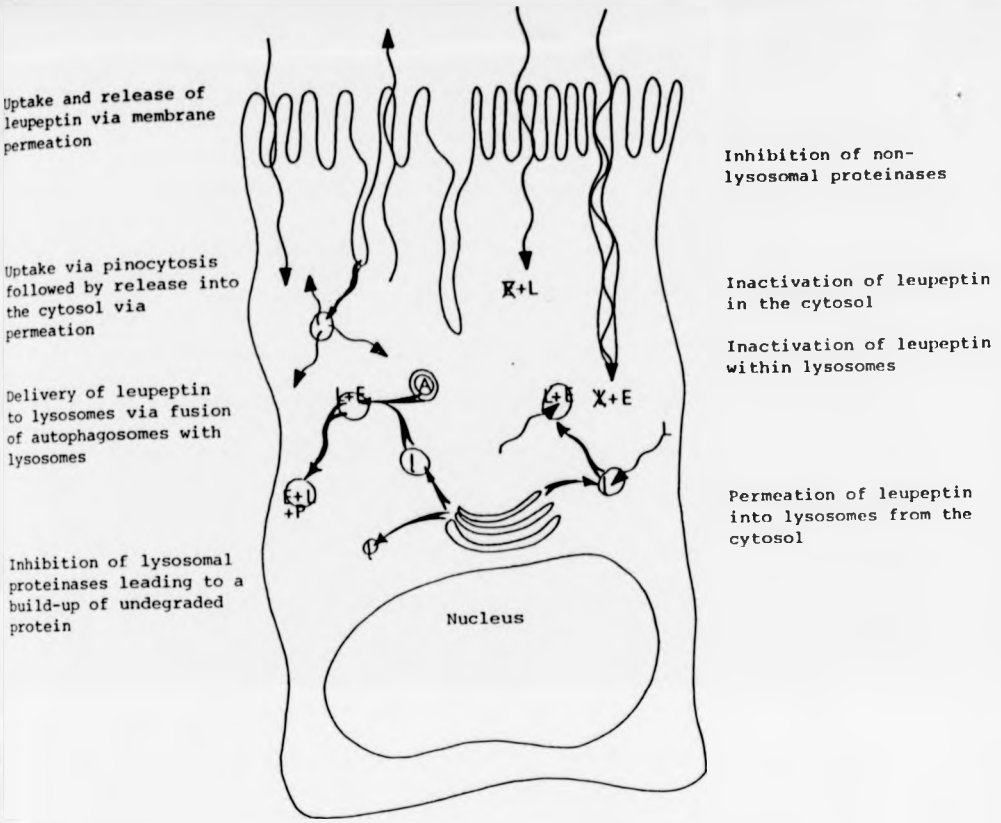



Fig. 9.2 Possible Intracellular Fates of Leupeptin MODEL B: Plasma Membrane Permeable Towards Leupeptin

Uptake of leupeptin would occur via both membrane permeation and pinocytosis, leading to delivery of leupeptin to the cytosol and lysosomes. Leupeptin may also enter lysosomes from the cytosol via direct permeation, microautophage uptake or by fusion with autophagosomes, pinosomes by permeation. Leupeptin may inhibit both lysosomal and non-lysosomal proteinases, and may become inactivated itself at either location.

## GENERAL DISCUSSION: POST-SCRIPT

After the experimental work and the majority of the writing of this thesis had been completed, a paper by Dennis & Aronson (1985) was published which reports in detail the uptake, subcellular distribution and metabolism of $^{3}$H-leupeptin by perfused rat liver and by rat liver *in vivo*. $^{3}$H-leupeptin was detected both by $^{3}$H-monitoring and by a papain-inhibition assay. [The latter was similar, in principle, to the chromogenic method used in this thesis. Active leupeptin was detected over the concentration range 0.09-0.23 µM (0.04-0.1µg/ml)]. Ion-exchange chromatography and paper electrophoresis were used to separate putative inactivation products, that could be detected (but not identified) by $^{3}$H-monitoring.

The main results reported by Dennis & Aronson (1985) are summarised below.

i) Uptake of Leupeptin

Uptake of $^{3}$H-leupeptin was measured in terms of the percentage of administered leupeptin that entered the liver plus bile during the experimental period. Over a 2h period, 35-50% of the leupeptin added to a perfusate was removed by the liver. About 49% of this radioactivity passed into the bile. At low concentrations (below 0.02mM) the rate of removal of leupeptin from the perfusate was greater during the initial 15min of perfusion. (This phenomenon was not observed for higher concentrations of leupeptin.) Uptake was not saturable over the range 0.001-0.4mM (0.44-176 µg/ml).

The uptake of leupeptin into the liver plus bile by a perfused liver was lowered by 25% in the presence of a mixture of eight amino acids (present at 500-1000 times the concentration of leupeptin), and slightly lowered (about 10%) by the tripeptide Leu-Arg-Leu (present at 140 times the concentration of leupeptin). Similarly, chemically-reduced leupeptin, ie. leupeptin alcohol, present at 200

times the concentration of leupeptin, diminished uptake by about 50%. Uptake into the liver plus bile was also inhibited by low temperatures and by the lysosomotropic weak base chloroquine. (The rate of uptake decreased linearly between 35 and 25°C; at 21°C only 5% of the added radioactivity entered the liver plus bile. Chloroquine, 0.2mM, inhibited total uptake by about 40%, but secretion into the bile was unchanged.)

ii) Subcellular Location of Leupeptin

The distribution of radioactivity in subcellular fractions of rat livers was measured at various time-intervals after injection of $^{3}$H-leupeptin into rats *in vivo*. The radioactivity in the heavy and light (ML) mitochondrial fraction increased from 15% (immediately after injection) to 49% after 90min; over the same time period, radioactivity in the supernatant decreased from 66% to 29%. [When $^{3}$H-leupeptin was added to a liver homogenate prior to fractionation, 75% of the radioactivity remained in the supernatant and only 4% became associated with the ML fraction.] When livers were treated with chloroquine (0.2mM) for 30min prior to perfusion with $^{3}$H-leupeptin, uptake into the ML fraction was inhibited by 75% and the percentage of radioactivity in the supernatant increased .

Although leupeptin appeared to accumulate within the mitochondrial fraction *in vivo*, radioactivity did not become associated with a mitochondrial fraction incubated *in vitro*.

iii) Metabolism of $^{3}$H-Leupeptin

Metabolism of $^{3}$H-leupeptin was assessed by measuring both its specific activity (in terms of % inhibition of papain per cpm), and the molecular nature of the radioactivity within a perfused liver. The radioactivity in the bile was shown to be intact leupeptin. However, 40% of the radioactivity in the liver itself was non-inhibitory, and was found to differ in characteristics from

$^{3}$H-leupeptin on ion-exchange and paper chromatography.

Although the exact uptake mechanism(s) of leupeptin was not determined the results were taken to suggest that uptake of leupeptin occurred mainly by direct diffusion across the plasma-membrane, although some leupeptin may enter the tissue by a more specific process. Pinocytosis was dismissed as a route of entry because fluid-phase pinocytosis would only account for about 2% of the volume of perfusate whose contained substrate entered the liver over a 2h period, and also because radioactivity became associated with the cytosolic fraction before the ML fraction. Inhibition of uptake by chloroquine and low temperature could indicate an energy requirement for uptake, although the latter inhibitor may have acted by decreasing membrane fluidity. Nearly 50% of the leupeptin that entered the tissue appears, intact, in the bile. This osbervation is not compatible with release of lysosomal contents (unless the leupeptin stimulated exocytosis) hence release by membrane permeation was considered more likely.

On comparing the results of Dennis & Aronson (1985) with those reported in this thesis some similarities are observed. However, a few results are very different. [Several experiments are peculiar to only one of the studies so do not permit a comparison of data.]

Similarities include the observation that uptake of leupeptin was inhibited by weak bases (ammonium chloride or chloroquine) and by low temperatures but was not saturable up to a concentration of 200µg/ml.

Divergent results include the rates of uptake of leupeptin. In the perfused liver, a greater percentage of $^{3}$H-leupeptin entered the liver than could be accounted for by fluid-phase pinocytosis alone, whereas in yolk sacs the rate of accumulation of $^{3}$H-leupeptin occurred at the same rate as fluid-phase pinocytosis. Another

difference between results was that, in the report of Dennis & Aronson, $^{3}$H-leupeptin did not become associated with liver lysosomes incubated *in vitro*. However, in the "Mego & McQueen" type experiments described in this thesis, it was observed that leupeptin was able to cross the membrane of isolated rat-liver lysosomes. Also, release of leupeptin from the liver into the bile was apparently greater than the release of leupeptin from yolk-sac tissue. (This might be expected for any low-molecular-weight substrate because exocytosis is known to occur in the liver.)

Several results of Dennis & Aronson (1985) had no equivalent in the work reported in this thesis, and the information provided by them will be discussed here.

i) In the perfused liver, large excesses of amino-acids or leupeptin analogues inhibited the uptake of $^{3}$H-leupeptin. It was suggested that this competitive inhibition could indicate that some uptake occurred by a specific process or by different transport mechanisms in different liver cell types. The main uptake mechanism was proposed to be direct permeation, because uptake of $^{3}$H-leupeptin alone was not saturable.

No control experiments to monitor the effects of the competitive inhibitors on the tissue itself were reported, and the concentration dependence of competition was not determined. Additional experiments may enable a more precise interpretation of these initial observations. It would also be interesting to carry out equivalent experiments in rat yolk-sac tissue to measure any inhibition of uptake of leupeptin by its analogues.

ii) The subcellular distribution of $^{3}$H-leupeptin that had entered the liver was determined in the experiments of Dennis & Aronson. (Unfortunately, this type of experiment was not possible in yolk-sac tissue because of difficulties in obtaining intact

subcellular organelles.)

Immediately after administration *in vivo*, very little of the radioactivity in the liver was associated with lysosomes (most was located in the cytosol), but, 90min after administration, the distribution had changed so that a greater percentage of the radioactivity was located in the lysosomes, rather than in the cytosol. The change in distribution was taken to suggest that leupeptin first entered the cytosol then accumulated in the lysosomes. This implies that uptake of leupeptin occurred by direct permeation across the plasma membrane into the cytosol, followed by entry into and accumulation within lysosomes. Chloroquine inhibited both uptake and accumulation within lysosomes.
It was suggested that this may be because a
low intralysosomal pH and/or active proton pump was necessary for leupeptin accumulation and/or uptake. Thus some leupeptin accumulation must be energy-dependent (although the report suggested that the most prominent route of entry was probably permeation through membrane pores).

However, an alternative explanation, other than uptake occurring entirely by membrane permeation, can be put forward; the results of Dennis & Aronson are compatible with at least a fraction of the leupeptin entering the tissue by pinocytosis (possibly adsorptive pinocytosis). Thus chloroquine (which inhibits pinocytosis) could inhibit uptake of leupeptin into lysosomes, as observed. (Any remaining uptake could occur by permeation directly into the cytosol.) The observation that leupeptin accumulates in lysosomes within 30min could also be attributed to a pinocytic mode of entry, as substrates taken up by pinocytosis are thought to reach lysosomes within about 10-15min.

The greater proportion of leupeptin in the cytosolic fraction at early time-points could be explained either by a proportion of uptake

occurring rapidly via direct membrane permeation, or by free $^{3}$H-leupeptin arising either from extracellular sites (by failure to be displaced by the flushing procedure) or possibly from lysed lysosomes. These possible alternative explanations are not discussed by Dennis & Aronson.

iii) Dennis & Aronson measured uptake of leupeptin in terms of the amount of radioactivity that accumulated in the liver tissue and in the bile. [The possibility of loss of leupeptin from the tissue back into the perfusion medium was not discussed, although back-diffusion would be expected if passive diffusion was the main route of entry. (Loss of leupeptin from yolk sacs back into the incubation medium was considered in the experiments described in this thesis, but no route of release equivalent to biliary secretion exists in yolk-sac tissue.)]

In perfused liver, leupeptin appeared in the bile in an inhibitory form a short time (within 15min) after addition to the perfusate. Moreover, the amount of leupeptin in the bile increased steadily over the 2h perfusion period, about 50% of the radioactivity cleared from the perfusate was located in the bile after this period. The presence of 0.2mM chloroquine (which inhibited total uptake the liver plus bile by about 40%) did not affect the amount of leupeptin secreted into the bile.

All $^{3}$H-leupeptin in the bile was assumed to have entered that compartment via an intracellular route through the liver tissue, and thus contributed to the total uptake by the tissue. However, it is possible that leupeptin may enter the bile via a paracellular route, by diffusion across the tight junctions between cells in the liver. This paracellular route has been investigated using markers such as sucrose (mol. mass 342) and inulin (mol. mass 5000) and can occur for much larger molecules such as horseradish peroxidase; Lowe *et al.*,

1985. (Substrates entering the bile via this route first appear within about 5min of addition to the perfusate.) Thus it appears likely that leupeptin (mol. mass approx 435) could also diffuse across tight junctions. Whatever the route by which intact leupeptin rapidly reaches the bile canaliculi in quantity, there appears to be a distinct possibility that intact leupeptin in this compartment will not be flushed from the liver and may mistakenly be assumed to be present in the cytosolic compartment when interpreting the results of subcellular fractionation experiments.

In conclusion, although the results of Dennis & Aronson (1985) were taken to suggest that the major route of uptake was via direct permeation of the plasma membrane, the data do not preclude other modes of uptake. In particular, uptake by pinocytosis (adsorptive) may be of more importance than originally assumed. The main arguments against a pinocytic mode of uptake were the rapid rate of clearance of leupeptin, and its subcellular distribution in liver tissue. However, as argued above, direct permeation across the plasma membrane may not be as great as proposed and a subcellular distribution similar to that observed could occur if pinocytosis was the main route of uptake by hepatocytes. Indeed, several of the reported results, such as those observed with chloroquine and at low temperatures, are equally compatible with a pinocytic mode of uptake.

It is possible that several different modes of entry contribute towards the uptake of leupeptin by liver tissue. These could include passive diffusion, mediated diffusion, and adsorptive pinocytosis. Different routes may be absent or be dominant in the different cell types, found in liver and yolk-sac tissue, so that there may be real differences between different tissues.

Dennis, P. A. & Aronson, N.N. Jr. (1985). Arch. Biochem. Biophys. **240**, 768-776.

## GENERAL DISCUSSION: POST SCRIPT II.

### NOMENCLATURE OF PROTEIN-DEGRADING ENZYMES.

Until fairly recently there has been widespread confusion over the nomenclature of proteolytic enzymes; this confusion is reflected in some of the terms used in this thesis. Recent publications (Barrett, 1980; Barrett & McDonald, 1985; Barrett & McDonald, 1986) have clarified the definitions and the following nomenclature and classifications are now accepted.

i) Peptidase: any hydrolase that cleaves peptide bonds. The substrate may have any number of amino acid residues and range in size from a small peptide to a protein. The term "peptidase" replaces the older term "protease".

ii) Exopeptidase: a hydrolase that cleaves peptide bonds at or near the ends of a polypeptide chain. Exopeptidases require the substrate to have a free carboxyl- or amino-terminal residue.

iii) Endopeptidase: a hydrolase that can cleave bonds distant from the ends of a polypeptide chain. [Some endopeptidases are also capable of cleaving bonds at the ends of a polypeptide chain, but these do not require free carboxyl- or amino-terminal residues.] An older expression, having the same meaning as "endopeptidase" is "proteinase".

Endoproteinases may be further classified according to their catalytic mechanism. The first three groups, now named after the active-site amino-acid residue most important for their mechanism of catalysis, are known as serine, cysteine (formerly "thiol") and aspartic (formerly "acid" or "aspartate") endopeptidases. A fourth group is the metallo-endopeptidases, which contain a metal ion as an integral part of the active site. [A fifth group of endopeptidases exists in which the

mechanism of catalysis is unknown.]

In order to conform with these definitions, the following modifications are required to the text of this thesis.

i) On pages 1 and 2 the terms "endopeptidase" and "exopeptidase" were defined as peptide hydrolases that act only on "peptides". This definition is not accurate: "peptidases" can act on both peptides and proteins.

ii) Also on pages 1 and 2 (and others e.g. pages 4, 8 9) the terms "endoproteinase" and "exoproteinase" were used. This nomenclature is not accepted; the terms "endopeptidase" and "exopeptidase" should have been used.

iii) The classification "thiol" was used (e.g. on pages 2, 4, 8, 185); the more recent nomenclature "cyteine" is to be preferred. Likewise, "aspartate" (p2) is better replaced with "aspartic".

iv) Throughout the thesis the term "proteinase" was used as a general term for all peptide bond hydrolases and should therefore have been replaced by the term "peptidase". However, as used in this thesis the term "proteinase" does in fact imply "endopeptidase", because endopeptidases generally initiate (and hence control) the total hydrolysis of proteins and because most peptidase inhibitors inhibit only endopeptidases. Thus the term "proteinase" (meaning endopeptidase), though an 'old-fashioned' word, is in fact correct in the context of this thesis, and was not altered in the final draft.

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# APPENDIX 1

## COMPUTER PROGRAMS

1.1. Uptake of $^{125}$I-PVP

1.2. Uptake of $^{125}$I-BSA$_{fd}$

1.3. Release of TCA-Soluble Radioactivity

Appendix 1.1

```
0010  DIM C£30
0020  REM KEW 125-IPVP PROGRAM,MODIFIED NOV.,1974
0027 REM FURTHER MODIFIED DECEMBER 1980 BY LEN
0030 DIMH(30),I(30),K(30),L(30),R(30)
0060  PRINT "NON-DIGESTIBLE PROG. ENTERED"
0070 % "EXPERIMENT CODE =";
0080 INPUT Z£
0090  PRINT "BACKGROUND IN CPM=";
0100  INPUT A
0110  PRINT "COUNTING TIME FOR EACH ML OF MEDIUM, SECS=";
0120  INPUT C
0130  PRINT "COUNTING TIME FOR EACH ML OF YS SOLUTION, SECS=";
0140  INPUT D
0150  PRINT "NO. OF POINTS IN PLOT=";
0160  INPUT G
0165  PRINT "NAME OF DATA FILE:";
0167  INPUT F1£
0170  PRINT "NAME OF REGRESSION FILE:";
0174  INPUT F2£
0176  FILES 125I-BSAfd,F1£,University of Keele,,F2£
0180  PRINT "NAME OF RESULTS FILE:";
0184  INPUT F3£
0186  FILES Keele,,F3£
0190  FOR X=1 TO G
0200  INPUT 125I-BSAfd, H(X),I(X),K(X),L(X),
0210  NEXT X
0220  FOR X=1 TO G
0230  LET M=(I(X)*60/C)-A
0240  LET Q=((K(X)*60/D)-A)*5
0250  LET N=M+Q/20
0260  LET R(X)=(Q*1000)/(N*L(X))
0265
0266
0270 % University of Keele,,H(X),",",R(X)
0280  NEXT X
0310  PRINT Keele,, " "
0315 % Keele,,"THIS IS EXPT CODED ";Z£
0320  PRINT Keele,, " "
0330 % Keele,,"INCUBATION TIME";"   PROTEIN IN Y.S. ";"   UPTAKE "
0332 % Keele,,"(HOURS)"
0340  PRINT Keele,,
0350  FOR X=1 TO G
0360 % Keele,,H(X),L(X),R(X)
0370  NEXT X
0380  STOP
```

Appendix 1.2

```
0020  REM PROTEIN PROGRAM ,REVISED OUTPUT ,NOV. 1974
0030  DIM E(12),H(12),I(12),J(12),K(12),L(12),S(12)
0040  DIM C£30
0045 DIM V£30
0060  PRINT "PROTEIN PROGRAM ENTERED"
0070 PRINT "EXPERIMENT CODE =";
0080 INPUT V£
0090  PRINT  "BACKGROUND IN CPM=";
0100  INPUT  A
0110  PRINT  "PERCENT SOLUBLES IN PREP=";
0120  INPUT  B
0130  PRINT  "COUNTING TIME MEDIUM TOTALS,SECS=";
0140  INPUT  C
0150  PRINT  "COUNTING TIME MEDIUM SOLUBLES,SECS=";
0160  INPUT  Z
0170  PRINT  "YS COUNTING TIME,SECS=";
0180  INPUT  D
0190  PRINT  "CORRECTION FACTOR FOR MEDIUM TOTALS=";
0200  INPUT  Y
0210  PRINT  "CORRECTION FACTOR FOR MEDIUM SOLUBLES=";
0220  INPUT  R
0230  PRINT  "NO. OF POINTS IN PLOT=";
0240  INPUT  G
0264  PRINT "NAME OF DATA FILE:";
0265  INPUT F1£
0266  PRINT "NAME OF REGRESSION FILE:";
0267  INPUT F2£
0269  FILES    125I-BSA_fd,F1£,University of Keele,,F2£
0270  PRINT "NAME OF RESULTS FILE:";
0272  INPUT F3£
0275  FILES Keele,,F3£
0277  FOR X=1 TO G
0280   INPUT    125I-BSA_fd, H(X),I(X),J(X),K(X),L(X),
0290  NEXT X
0300  FOR X=1 TO G
0310  LET M=(I(X)*60/C-A)*Y
0320  LET N=(J(X)*60/Z-A)*R
0330  LET O=N-(M*B/100)
0340  LET Q=((K(X)*60/D)-A)*5
0350  LET P=(M-N)+O/2
0360  LET F=((10*O)+Q)*1000
0370  LET E(X)=(Q*1000)/(L(X)*P)
0380  LET S(X)=F/(L(X)*P)
0385
0387 % Keele,,
0390 % University of Keele,,H(X),",",S(X)
0400  NEXT X
0405 % Keele,,
0410 % Keele,,"GOOD MORNING! THIS IS EXPT CODED ";V£
0430  PRINT Keele,, " "
0435
0440  PRINT Keele,, " "
0445 % Keele,,
0450  PRINT Keele,, " INCUBATION ","PROTEIN","MICROLITRES","
UPTAKE"
0460  PRINT Keele,, "TIME (HOURS)"," IN YS "," PER MG YS "
0465 % Keele,,
0470  FOR X=1 TO G
0480 % Keele,,H(X),L(X),E(X),S(X)
0490  NEXT X
0500  STOP
```

Appendix 1.3

```
      DIMENSION COUNTS(100),CPM(100),T(100),SIGC(101)
      DIMENSION FNAME(20)
      DATA SPACES/4H    /,FSTOP/4HSTOP/
    1 WRITE(2,*)"INPUT DATA FILENAME"
      READ(1,110)(FNAME(I),I=1,20)
  110 FORMAT(20A4)
      CALL CONECT(0,3)
      IF (FNAME(1).EQ.FSTOP)GOTO 50
      IF (FNAME(1).EQ.SPACES)GOTO 4
      CALL CONECT(FNAME,3)
      GOTO 6
    4 CALL CONECT(4H*   ,3)
    6 READ(3,*)N,WEIGHT,ITIME,IBACK
      SIGC(1)=0.0
C     READ THE COUNTS
      DO 10 I=1,N
   10 READ(3,*)COUNTS(I)
C     CORRECT COUNTS FOR TIME,BG,CF
      DO 20 I=1,N
      CPM(I)=((COUNTS(I)*60.0/FLOAT(ITIME))-FLOAT(IBACK))*1.2065
   20 SIGC(I+1)=SIGC(I)+CPM(I)
C      CCALCULATE TOTAL COUNTS
      WRITE(6,105)(FNAME(I),I=1,20)
  105 FORMAT(1H1,12H DATA FROM ,20A4//)
      WRITE(6,115)
  115 FORMAT(1X,3HNO.,3X,6HCOUNTS,7X,3HCPM,9X,4HSIGC,7X,5HTOTAL/)
      DO 30 I=1,N
      T(I)=((10.0*CPM(I)+SIGC(I))/WEIGHT)
      WRITE(4,100)I,COUNTS(I),CPM(I),SIGC(I),T(I)
   30 WRITE(6,100) I,COUNTS(I),CPM(I),SIGC(I),T(I)
  100 FORMAT(1X,I3,4(3X,F9.0))
      GOTO 1
   50 STOP
      END
```

# APPENDIX 2

## Preparation of Yolk-Sac Tissue for Assay of Active Leupeptin

## APPENDIX 2

### Preparation of Yolk-Sac Tissue for Assay of Active Leupeptin

Efficient extraction of leupeptin from tissue was important for the quantitative estimation of the amount of leupeptin that had accumulated within tissue during incubation. Several different methods of tissue disruption were tested for their suitability. (Suitable methods were defined as those that were quick and effective, and that gave a high recovery of leupeptin). Tissue disruption was assessed qualitatively by observing the amount of tissue debris remaining at the end of the disruption procedure. The percentage recovery of leupeptin was estimated by adding a known amount of leupeptin to intact, unincubated tissue then disrupting the tissue and assaying its leupeptin content using the fluorometric method described in Section 5.2.1.

The following methods of tissue disruption were tested.

**i) Homogenization** The precise methods of homogenization used for each experiment are given in the relevant chapters. Greatest tissue disruption was achieved using a hand-held ground-glass-on-ground-glass tissue homogenizer, particularly when tissue was homogenized from the deep-frozen (-20°C) state.

Homogenization using a Teflon-on-glass, Potter-Elvehjem type homogenizer did not disrupt tissue efficiently; best results using this homogenizer were obtained when at least three yolk sacs were homogenized together

[Recovery of leupeptin from homogenized tissue that was either assayed directly or treated with TCA (which precipitated cell proteins leaving leupeptin in the soluble fraction) is discussed in Sections 4.3.4 and 5.3.2].

Leupeptin was stable on storage at -20°C in a yolk-sac

homogenate or the TCA - soluble fraction of a homogenate.

**ii) Grinding Under Liquid Nitrogen** Yolk sacs were immersed in liquid nitrogen in a stoneware mortar. Once frozen, the tissue was ground with a pestle (also at liquid nitrogen temperature) then the nitrogen allowed to evaporate. The powdery, frozen residue was quickly transferred to a small vial, and distilled water added.

The method was not considered suitable for general use because quantitative transfer of tissue from mortar to vial was difficult, and fairly large quantities of liquid nitrogen were required.

**iii) Collagenase Disintegration** Collagenase (Sigma Types IV or V) was used at concentrations of 1,5 and 10mg/ml in Tris buffer (0.1M, pH 7.5) containing calcium chloride (10mM). Yolk sacs were placed singly in small vials and finely chopped with scissors. Collagenase solution (0.5ml) was added and the vials incubated at 37°C, with occasional vortexing. Tissue became fully disrupted within 12h at all concentrations of collagenase tested.

The method was convienient in that it was less labour intensive than homogenization or grinding. However, leupeptin was inactivated during incubation with collagenase solution (only about 10% of the added leupeptin remained after 6h incubation with collagenase, Type IV, at 37°C) therefore the method could not be used to disrupt tissue containing leupeptin prior to assay.

**iv) Digestion in Sodium Hydroxide** Yolk sacs were digested in sodium hydroxide solution (1M, 0.5ml per yolk sac) at 37°C. Digestion to a clear solution took approximately 1h. However, leupeptin lost activity rapidly when incubated with sodium hydroxide, therefore the method was unsuitable for use in preparing leupeptin - containing tissue for assay.

# APPENDIX 3

## Preparation of Yolk-Sac Tissue for Assay of $^{3}H$- and $^{14}C$-Radioactivity

## APPENDIX 3

### Preparation of Yolk-Sac Tissue for Assay of $^{3}H$- and $^{14}C$-Radioactivity

It was necessary to disrupt yolk-sac tissue prior to assay of $^{14}C$- and $^{3}H$-radioactivity, both to improve counting efficiency and to enable samples to be removed for assay of protein content. The method of disruption used should give a homogeneous susupension or solution with a high counting efficiency, so that counts are reproducible both between tissue samples and over time. Several methods were tested for their suitability, by comparing the observed counts in a "spike" of radioactivity added to both unincubated tissue and water.

**i) Neutralised Sodium Hydroxide** Tissue was first digested in sodium hydroxide (0.5M, 2ml/yolk sac) for 4h at 37°C, then the solution neutralised with an equal molar quantity of nitric acid and the volume made up to 5ml with distilled water. Duplicate samples (0.5ml) were mixed with scintillation fluid, allowed to stand overnight, then assayed for radioactivity.

This method was found to give poor reproducibility between tissue samples. Some vials contained a precipitate that reduced the counting efficiency relative to other spiked tissue and to water.

**ii) Commercial Tissue Solubilizer** Tissue solubilizer (NCS, 1ml/yolk sac) was added to the tissue and the tissue digested overnight at 37°C. A sample (0.5ml) of the digest was mixed with scintillation fluid (4ml), allowed to stand overnight, then counted.

The method was not suitable for use with experimental tissue because the digest could not be used in the protein assay.

**iii) Collagenase** Collagenase solution (Worthington Type CLS III 1mg/ml dissolved in Tris buffer, 0.1M pH 7.5, containing calcium chloride, 10mM) was added to the tissue (0.5ml/yolk sac) and incubated overnight at 37°C. A sample (0.4ml) of the resulting cell suspension was mixed with scintillation fluid (4ml), allowed to stand overnight, then counted.

The method gave good counting efficiency relative to water (mean 97%) but was not ideal because of the relatively high cost of collagenase, long period of time required for disruption, and additional calculations required to determine the protein content of the tissue alone.

**iv) Sodium Hydroxide** Tissue was digested in a small volume of sodium hydroxide (1M, 1ml/yolk sac) over a period of 2h at 37°C. A sample (0.5ml) of the digest was mixed with scintillation fluid (4ml), and either counted immediately or allowed to stand for a period of 2h - 9 days before counting.

Immediately after addition of scintillation fluid, the number of counts observed from spikes of $^{14}C$- sucrose and $^{3}H_2O$ in a yolk-sac digest was greater than in water (counting efficiency relative to water was about 110%), probably because of chemiluminesence. However, the number of counts observed in the digest when $^{14}C$- surcrose was used as the spike decreased markedly over the 9 day monitoring period, the relative counting efficiency falling to 50% on standing overnight and 30% after 5-9 days. A less marked drop occurred when $^{3}H_2O$ was used as the spike, the realative counting efficiency falling from 115% to 105%. Because chemiluminesence and loss of observed $^{14}C$ counts occurred the method was not considered suitable for the preparation of experimental tissue for assay.

**v) Homogenization** Yolk sacs were homogenized (from frozen) in distilled water (1ml/yolk sac) in a hand-held ground-glass homogenizer. Duplicate samples (0.4ml) were mixed with scintillation fluid (4ml) and allowed to stand overnight before counting, (except when reproducibility of counts over time was investigated, when samples were counted immediately then at intervals over 9 days).

This method was used for all experimental tissues. The method gave good reproducibility over time for both $^{14}C$ and $^{3}H$ counts, and counting efficiency was high and fairly reproducible. The protein content of the homogenate was readily determined.

# APPENDIX 4

## Analysis of Leupeptin Degradation Products

## APPENDIX 4

### Analysis of Leupeptin Degradation Products

The separation and possible identification of leupeptin degradation products was desirable for several reasons, one of the most important being in the characterisation of material released from leupeptin-loaded yolk-sac tissue. If the material released was found to be mainly intact leupeptin, this would suggest that leupeptin is capable of permeating out of the cell across the plasma membrane. If, however, it was found that mainly degradation products of leupeptin were released, it may have been possible to calculate the total uptake of leupeptin (rather than simple tissue-accumulation) by estimating the amount of degradation products in the incubation medium and adding this to the amount of material detected in the tissue after each period of incubation. The method of separation used need not identify the degradation products produced, but would ideally be quick and simple (similar, for example, to the addition of TCA to precipitate, and thus separate, an intact protein from its degradation products). It would also need to be both quantitative and reliable for small quantities of material (such as those present in re-incubation medium or in incubation medium containing a high concentration of intact leupeptin, but only small quantities of degradation product).

If a method were found to identify inactivation and/or degradation products, metabolism of leupeptin within tissue could have been monitored. This, in turn, may have provided an explanation for the observed loss of active leupeptin, but not $^{3}H$-radioactivity derived from leupeptin, from yolk-sac tissue.

Several potential methods of separating leupeptin from its putative degradation products were tested. [$^{3}H$-leupeptin was used

for all experiments because any degradation product(s) could be readily detected by beta-counting]. Experimental details, together with the basis of separation and results for each method are given below.

**i) Paper Chromatography**

Paper chromatography separates materials mainly on the basis of their relative affinity (ie partition) between a mobile solvent phase and a stationary water phase (that is absorbed to the paper). Adsorption of the material itself to the paper may also contribute to separation.

Two solvent systems were tested for use in the separation of leupeptin from putative degradation products. These were:

a) A mixture of butanol: glacial acetic acid: water (volume ratio 12:3:5), which is reported to give good separation of amino acids. The $R_f$ value of leucine in this system is reported to be 0.79 (Browning, 1973).

b) A mixture of propan-1-ol : water (volume ratio 7:3), which was used by Kando et al. (1969) to demonstrate the purity of leupeptin. Leupeptin has an $R_f$ value of 0.9-0.95 in this system.

$^3$H- leupeptin stock solution and re-incubation medium containing material released from $^3$H-leupeptin-loaded yolk sacs were loaded onto sheets of filter paper (Whatman No. 1) as small spots. The paper was placed in tanks containing one of the above solvent systems (approx. 200ml) and developed for 4h. The chomatographs were dryed and cut into horizontal strips lcm wide, which were placed in scintillation vials, scintillation fluid was added and the vials counted for beta-radioactivity (after having stood overnight).

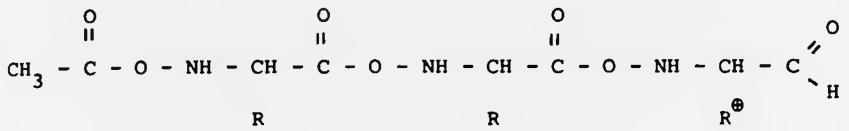
Results were similar for both solvent systems. All radioactivity travelled with the solvent front (ie $R_f$ value 1.0). No secondary

peaks were detected for leupeptin-derived radioactivity released from yolk sacs, suggesting either that the method was not capable of separating degradation products from leupeptin, or that no such products were present, or that the method was not sufficiently sensitive to detect the small amount of degradation product loaded onto the paper.

**ii) Cation-Exchange Chromatography**

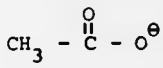
Cation-exchange resins contain acidic, negatively-charged groups that bind positively charged ions or molecules, but allow negatively-charged material to remain free in solution. Bound material may be removed from the resin by displacement with other ions, provided the displacement ions have a greater affinity for the resin. It was hoped that leupeptin could be separated from at least some of its degradation products on the basis of charge differences related to the structures which are given over the page.

Acetyl - Leucyl - Leucyl - Arginal — Intact (Leupeptin)



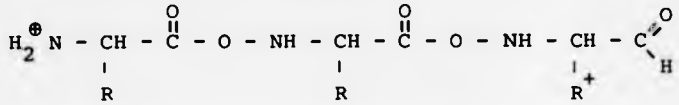
Net charge +1

Acetate



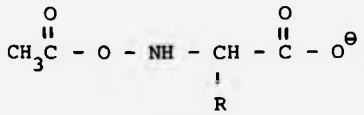
Net charge -1

Leucyl - Leucyl - Arginal



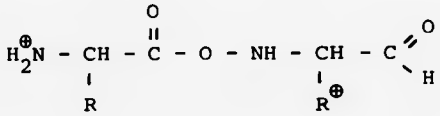
Net charge +2

Acetyl - Leucine



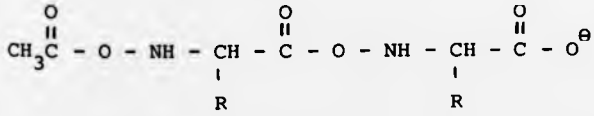
Net charge -1

Leucyl - Arginal



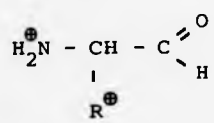
Net charge +2

Acetyl - Leucyl - Leucine



Net charge -1

Arginal



Net charge +2

In the absence of any other strongly binding cations intact leupeptin should bind to the cation-exchange resin, but only 50% of leupeptin degradation products should bind. The proportion of radioactivity bound to the resin should therefore provide an indication of the amount of degraded leupeptin in the sample.

The ion-exchange resin used was Dowex 50W x 4, which contains the functional group $-SO_3^-$ (a strong acid). It was prepared for use by first washing with methanol (to remove organic impurities), then with hydrochloric acid (followed by distilled water) to remove any positive ions that were bound, then with sodium hydroxide (followed by water) to ensure all functional groups were present as $SO_3Na$). After addition of the sample, un-bound material can be removed with distilled water and bound material with hydrochloric acid or excess sodium hydroxide.

Two methods of mixing sample with resin and measuring the proportion of bound radioactivity were tested.

a) Batch Method: Dowex 50W x 4, suspended in distilled water (0.1ml), was mixed with $^3H$- leupetin stock solution and allowed to stand approximately 5min. The supernatant was then removed and the Dowex washed with distilled water (2 x 0.5ml). The supernatant, washings and Dowex were all counted for radioactivity. In some experiments, a portion of the Dowex was washed with hydrochloric acid before counting.

b) Column Method: Dowex 50W x 4 slurry in distilled water was used to pack a small column (approximately 0.7cm diameter by 2cm length). $^3H$-leupeptin stock solution was loaded onto the column and eluted first with water (about 15ml) then with sodium hydroxide (1.0M, about 13ml). Eluant fractions were collected and counted for radioactitivty. Fractions containing sodium hydroxide were neutralised with hydrochloric acid before addition of

scintillation fluid.

The results obtained using the batch method (a) indicated that most radioactivity was detected in the supernatant and wash fractions; only about 2% of the radioactivity loaded was detected in the resin itself. However, when a portion of Dowex resin was washed with hydrochloric acid, the washings contained a greater number of counts than the unwashed Dowex, suggesting that not all $^{3}$H-radioactivity bound to the resin was detected. (This was verified by counting a "spike" of intact $^{3}$H-leupeptin of known radioactivity in the presence of Dowex; only 33% of the radioactivity was detected). It was therefore decided to try method (b). Only about 8% of the loaded radioactivity was eluted with distilled water, indicating that most leupeptin remained associated with the resin. Sodium hydroxide released 70-80% of the loaded radioactivity from the column. However, the method was not considered suitable for general use because of the large dilution of sample necessary during elution (the small number of counts present in re-incubation medium would not be detectable after such dilution). Also, problems were encountered when assaying the radioactivity in samples containing alkali because of quenching and chemiluminescence.

**iii) Paper Electrophoresis**

In addition to the effects of applied voltage, the migration rates of compounds during paper electrophoresis depend mainly on the net charge on compounds. (The latter in turn may depend on the pH of the electrophoresis medium, and on the type of buffer used) It was hoped that the difference in charge between leupeptin and its potential degradation products (discussed in the previous section) would provide a basis for separation. Leupeptin degradation would be detected by the appearance of negatively-charged fragments that

migrated towards the anode.

Strips of Whatman No. 1 paper were wetted with buffer (M, pH) and stretched across the apparatus. The current was switched on for approx. 15min, then (with the current off) either leupeptin stock solution or incubation medium that may contain degradation products released from yolk sacs was loaded across the width of the centre of the strip. The paper was then run at 400v and 17mA for 10min, then the paper removed, dried, and cut into strips 1cm wide for 10cm either side of the loading band. The strips were placed in vials, scintillation fluid added, and the vials counted for radioactivity (after having stood overnight).

Typical results are shown in Figs. IVa and IVb. Leupeptin stock solution gave a single peak that had migrated only a very short distance towards the cathode. Medium containing leupeptin that had been incubated with yolk sacs gave two peaks, one in a similar position to that of stock leupeptin, the other almost out of the monitored range nearest the anode. (The peaks were located in these positions even when the period of electrophoresis was increased) The radioactive moiety that gave rise to the second peak was clearly negatively charged, suggesting that it may have been a fragment of leupeptin containing the acyl group. Appropriate markers (such as labelled acetic acid, N-acetyl-Leucine) would be required for positive identification.

Paper electophoresis thus seemed to be a potential method of separating leupeptin degradation products from intact leupeptin, however further work would be required before it could be used as a reliable quantitative test. For example, complete recovery of all radioactivity after electrophoresis should be ensured. It was expected that, if negatively-charged degradation products had been generated, positively-charged fragments would also be produced,

however none was detected.

The positively charged degradation products may have had a similar mobility to intact leupeptin (in which case quantification of the proportion of intact leupentin and degradation products may be difficult). Ideally, the separation of the possible degradation products would have been established using labelled markers.

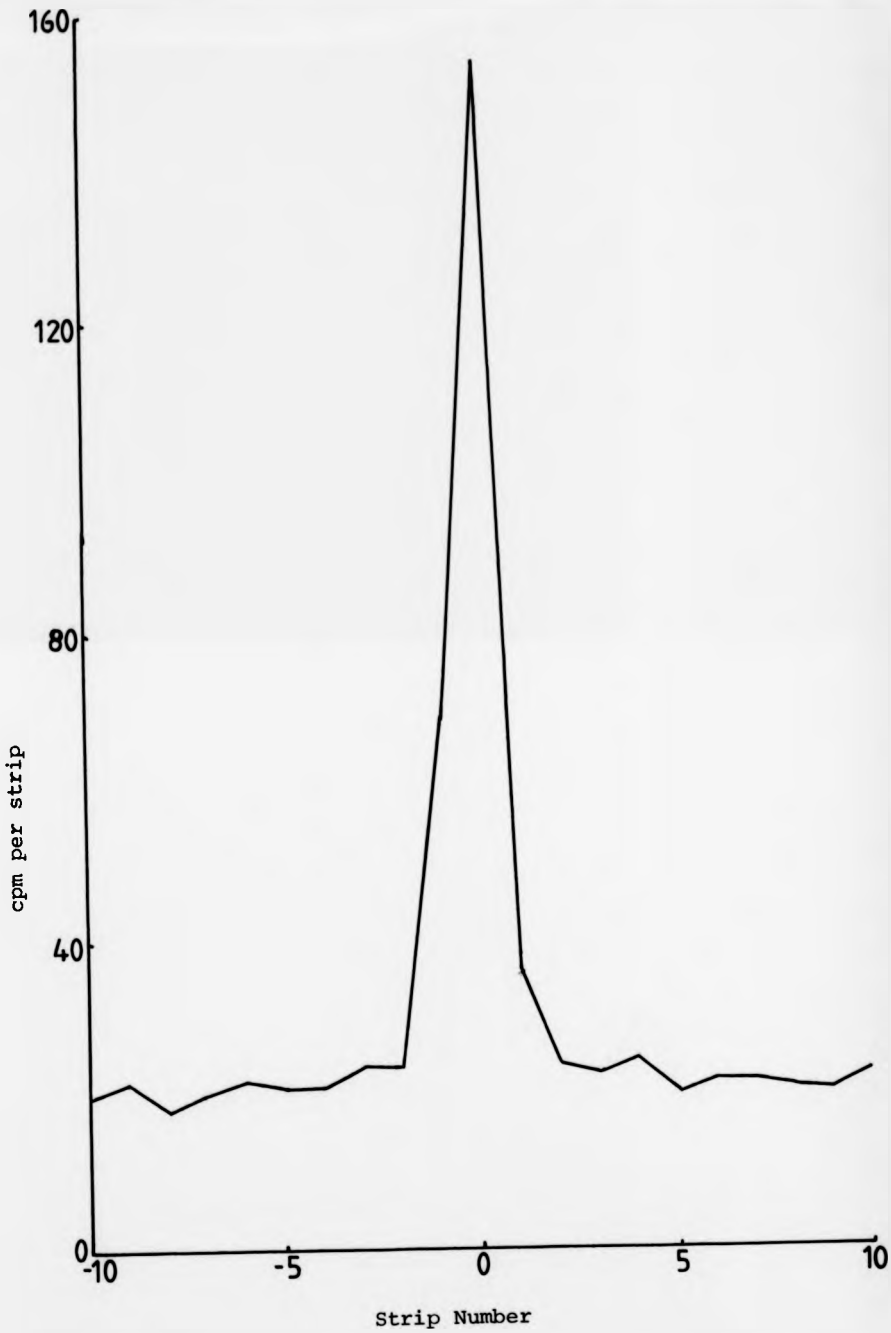



Fig. IVa Paper Electrophoresis of $^{3}$H-Leupeptin Stock Solution

Experimental details as given in Appendix 4

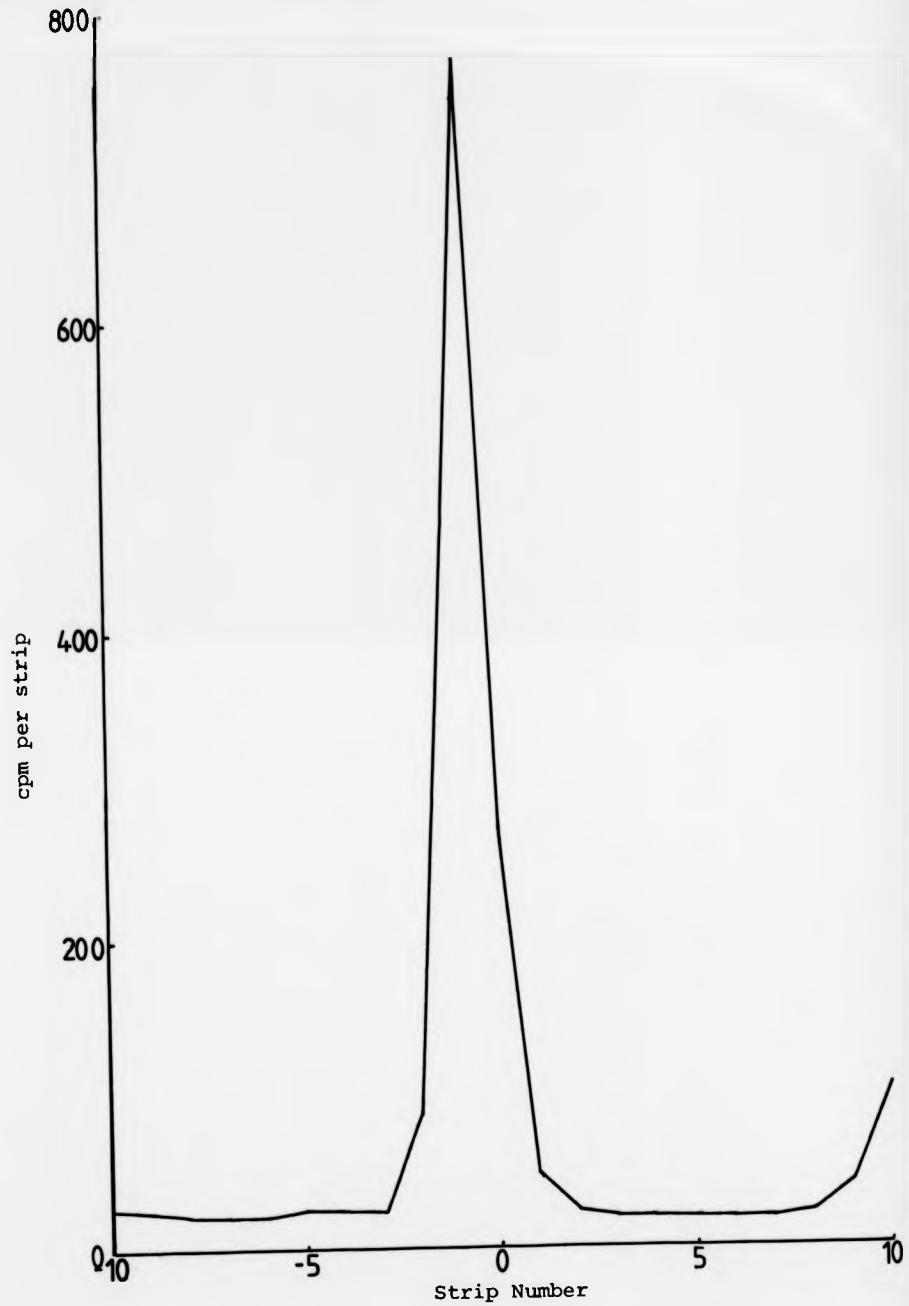



Fig. IVb Paper Electrophoresis of Incubation Medium Containing $^{3}$H-Leupeptin

Yolk sacs were incubated in medium containing $^{3}$H-leupeptin for approximately 5h, then a sample of the incubation medium used for paper electrophoresis, as described in Appendix 4.